

**DISTRIBUTION AND FREQUENCY OF MYELOID AND T CELL POPULATIONS IN
THE SMALL INTESTINE OF NEWBORN AND WEANED CALVES**

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ABSTRACT

The development of mucosal dendritic cells (DCs) in cattle is poorly understood and an analysis of myeloid cells in the bovine small intestine is required to increase our knowledge in this area. The phenotype, frequency and distribution of mucosal myeloid and lymphoid lamina propria leukocytes (LPL) and intraepithelial leukocytes (IEL) in the ileum and jejunum of newborn calves (3-5 weeks old) were analyzed using flow cytometry and immunohistochemistry (IHC). LPL and IEL were isolated through the use of chemical and enzymatic incubations. Co-staining with a CD45-specific monoclonal antibody allowed us to exclude all non-leukocytic cells from our analysis of IEL and LPL. The morphology of CD45⁺CD11c⁺MHC Class II⁺ cells isolated from the lamina propria (LP) of ileum and jejunum showed myeloid characteristics, validating the use of CD11c and MHC Class II co-expression to identify myeloid cells.

Regional differences in the frequency and number of leukocytes isolated from the IEL and LP compartments of the ileum and jejunum were analyzed in newborn calves. The CD11c^{Hi}CD14⁺ and CD335⁺ NK cell populations were significantly more abundant in the ileum than the jejunum. IHC was then used to identify the distribution of myeloid cells within the intestine. This analysis confirmed the presence of a variety of myeloid cell populations within the LP. Furthermore, CD11c⁺ cells were uniquely distributed within the jejunal, but not the ileal IEL compartment. In contrast, CD11b⁺ cells were present in the ileal, but absent from the jejunal, IEL compartment. A comparison of myeloid cell populations isolated from jejunum and blood identified distinct mucosal DC populations, such as CD11c⁺CD13⁺ cells, which were present in the jejunum but absent from blood.

The phenotype, frequency and distribution of IEL and LPL in the ileum and jejunum of weaned calves (6 months old) were then investigated. Significant regional differences were observed when comparing mucosal T cell populations with CD8⁺ and $\gamma\delta$ T cells more abundant in the ileum and CD4⁺ T cells more abundant in the jejunum. Proportionally, there were no significant differences between the frequency and number of myeloid populations in the two regions. IHC was, once again, used to confirm these unique distributions of cells within each region. CD11b⁺ cells were present in the LP of both the ileum and jejunum, although a small number of CD11b⁺ cells were found in the ileal epithelium. CD4⁺ T cells were restricted to the LP, while CD8⁺ and $\gamma\delta$ T cells were restricted to the IEL compartment.

Significant age-related changes were observed when comparing mucosal leukocyte populations in the ileum and jejunum of newborn and 6 month old calves. In the ileum there was an age-related enrichment of CD8⁺ and $\gamma\delta$ T cells, while in the jejunum there was enrichment in CD4⁺ and CD8⁺ T cells. In contrast, total myeloid (CD11c⁺MHC Class II⁺) cells number remained unchanged but there was a significant age-related enrichment of DC subpopulations (CD13, CD26, CD205).

In conclusion, the ileum and jejunum of the newborn calf was populated by diverse myeloid subpopulations, some of which were distinct from myeloid subpopulations identified in blood. Furthermore, the total number of CD11c^{Hi}MHC Class II⁺ myeloid cells isolated from a 10 cm segment of intestine did not change with age. If neonatal DCs are functionally equivalent to DCs present in weaned calves then the neonatal mucosal immune system appears to have an equivalent capacity to acquire and present antigens acquired from diet, commensal microflora, or pathogens. The one limitation to this conclusion may be the marked difference in the distribution of intraepithelial DC and macrophage distribution when comparing newborn and weaned calves.

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LIST OF ABBREVIATIONS

APC	Allophycocyanin
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BHV-1	Bovine herpes virus 1
BRSV	Bovine respiratory syncytial virus
BVDV	Bovine viral diarrhea virus
CCR	Chemokine receptor
CRC	Canada Research Chair
CD	Cluster of differentiation
CD	Crohn's disease
CDP	Common dendritic cell precursor cell
CIHR	Canadian Institutes for Health Research
CMF-HBSS	Calcium & magnesium free Hank's balanced saline solution
DC	Dendritic cell
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence activated cell sorting
FAE	Follicle-associated epithelium
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter

FP	Fungiform papillae
GALT	Gut-associated lymphoid tissue
GIT	Gastrointestinal tract
HSC	Hematopoietic stem cell
IEC	Intestinal epithelial cell
IEL	Intraepithelial leukocyte
IF	Interfollicular
Ig	Immunoglobulin
IHC	Immunohistochemistry
LC	Langerhans cells
LP	Lamina propria
LPL	Lamina propria leukocyte
mAb	Monoclonal antibody
MALT	Mucosa-associated lymphoid tissue
MDP	Monocyte/macrophage and dendritic cell precursor cell
ME	Mucosal epithelium
MHC	Major histocompatibility complex
NLR	NOD-like receptor
NK	Natural killer
omDC	Oral mucosa dendritic cell
PBMC	Peripheral blood mononuclear cells
PBSA	Phosphate buffered saline
PDC	Plasmacytoid dendritic cell

PE	Phycoerythrin
PP	Peyer's patch
RPM	Revolutions per minute
SCFA	Short chain fatty acids
SED	Subepithelial dome
SLIT	Sublingual immunotherapy
SSC	Side scatter
TLR	Toll-like receptor
VFA	Volatile fatty acids
VIDO	Vaccine and Infectious Disease Organization

CHAPTER 1

LITERATURE REVIEW

1.1 Mucosal Immunity

The skin is an important first line of defence to protect against infectious disease but mucosal surfaces cover 300 m² versus the 2 m² for human skin [1]. This massive mucosal surface area provides many opportunities for pathogens to enter the body. The mucosal immune system is an integral part of the immune system as it provides a location for extensive interaction between the internal and external environments. The role of the systemic immune system is to direct specific attacks against invading pathogens. In contrast, the role of the mucosal immune system is to limit the number of foreign antigens that can invade and reach the systemic immune system. By virtue of its location, the mucosal immune system encounters many times more foreign antigen than the systemic immune system.

The mucosal immune system has also developed a well-regulated system that functions to prevent non-pathogenic organisms and dietary antigens from eliciting destructive immune responses. If the mucosal immune system did not differentially regulate immune responses to pathogens and commensal microflora there would be chronic inflammation at mucosal surfaces with disruption of critical biological functions. Therefore, the mucosal immune system has developed mechanisms to differentially regulate responses to pathogenic versus non-pathogenic microorganisms and dietary antigens. It may be said that the mucosal immune system functions more to suppress and down-regulate immune response than promoting the activation of pro-inflammatory and destructive immune response[2].

The organized lymphoid tissues of the mucosal immune system are collectively known as the mucosa-associated lymphoid tissue (MALT). The gut-associated lymphoid tissue (GALT) is

an important component of the MALT and the GALT spans the entire length of the gastrointestinal tract (GIT). Within the MALT there are further regional subdivisions, such as the lung, nose, larynx, eyes, and reproductive tract. I will focus mainly on the makeup of the GALT which includes the tonsils, PP, and all other organized lymphoid tissues located throughout the GIT.

One of the primary functions of the mucosal immune system is to provide a barrier separating the interior and exterior environments. Without this barrier microorganisms and pathogens could freely enter the body. Enzymes throughout the MALT, such as pepsin, trypsin and chymotrypsin break down macromolecules to smaller molecules. This allows for nutrients to be absorbed but also renders immunogenic proteins nonimmunogenic. Other protective elements include bile salts and extreme gastric pH that limits viable pathogens entering the GIT. Trefoil factors repair any damage to the mucosal barrier and strengthen the epithelial barrier to prevent pathogen invasion [3].

The mucosal epithelium is composed of five cell types: absorptive epithelium, goblet cells, Paneth cells, M cells and crypt epithelium. The mucosal epithelium also uses a sticky glycoprotein mucous layer to protect epithelial cells from the dangers of the lumen[2]. Many pathogens attempting entry through or between epithelial cells are trapped in the glycocalyx and simply excreted through peristalsis and defecation. Goblet cells produce mucin, which is similar to the glycocalyx and lines epithelial cells[4]. The glycocalyx and mucin work in concert to prevent antigens or pathogens from crossing the epithelial layer. This is known as non-immune exclusion.

Another type of specialized mucosal epithelial cell is called the microfold (M) cell. An M cell is an epithelial cell that has a shorter glycocalyx and is located in the epithelium overlying PP. M cells are specialized to sample and transport luminal antigens into the underlying

lymphoid tissue[5]. M cells pass antigens through the epithelial layer to the underlying macrophages, which then carry antigen into the PP for the induction of an acquired immune response[6].

Epithelial cells also maintain tight junctions between cells. This prevents macromolecules and microorganisms from crossing the epithelial cell barrier. Only small ions are able to pass through these tight junctions. Abnormal tight junctions have been postulated to be a contributing factor in food allergies[7]. Loss of tight junction integrity may result in more antigens being able to penetrate through the epithelial layer and elicit an immune response. Various studies have hypothesized that in human neonates tight junctions are relaxed to allow more antigens into the lamina propria (LP) [8].

Epithelial cells can also sample antigens in the lumen and directly present antigen T cells in the LP [9]. Epithelial cells express surface molecules that allow them to interact with T cells, such as MHC classes I and II, E cadherin, and the gp180 protein[10]. E cadherin binds to the integrin $\alpha E\beta 7$ found on CD8⁺ intraepithelial leukocytes (IEL)[11]. Epithelial cells also play an important role in regulating T cell DC development through the production of cytokines such as thymic stromal lymphopoietin[12]. Defects in this system can lead to inflammatory bowel disease and food allergies.

Antimicrobial peptides (AMPs) play an important part in regulating the growth and colonization of microflora in the intestine. AMPs are part of the innate immune system and do not discriminate between commensal and pathogenic bacteria. AMPs are molecules secreted predominately by Paneth cells, but can also be produced by goblet cells, epithelial cells, macrophages and neutrophils[13]. Types of AMPs include defensins, cathelicidins, C-type lectins and metal withholding antimicrobial molecules. Defensins act against all bacteria and

certain viruses and fungi by disrupting their membranes, leading to loss of function and death. The expression of defensins by intestinal cells depends upon activation by toll-like receptors (TLRs) or NOD-like receptors (NLRs), which lead to a signalling cascade[14]. Cathelicidins also act by disrupting the membrane of bacteria and fungi. They can also induce a Th1 response and act as a chemo-attractant for T cells and macrophages[15]. C-type lectins, such as RegIII γ , bind to the carbohydrate backbone of peptidoglycan and can destroy Gram-positive bacteria[16]. Metal withholding antimicrobials are able to sequester metals required by certain bacteria, such as zinc and iron[17]. AMPs are also associated with immune regulation, attraction of immune cells, recruitment of macrophages and DCs, wound healing, and angiogenesis.

Intraepithelial leukocytes consist of approximately 90% CD8⁺ and $\gamma\delta$ T cells in mice, although in humans only CD8⁺ T cells are present in this immune compartment[18]. In cattle IEL consist of CD8⁺ and $\gamma\delta$ T cells, as well CD11b⁺ and CD11c⁺ myeloid cells. It is believed that the IEL do not actively take part in luminal sampling, but rather may be present to effect cytotoxic responses against epithelial cells that have undergone some form of stress or infection[19].

The LP of the intestine is a region of diffuse lymphoid tissue that contains a diverse array of cells. Histologically, the LP is situated between the epithelium and the muscularis mucosa, and is made up of connective tissue that contains leukocytes and other cells. The LP contains T cells, B cells, DCs, NK cells, macrophages, and a variety of granulocytes[20]. Within the LP there are many different distinct DCs which perform a variety of distinct functions[21]. The large number of cells in this region indicates that it is a site where antigen crosses the epithelial layer. This is done by professional antigen-presenting cells (APC), such as DCs[22]. Most of the T cells in the LP are CD4⁺ T cells, which is in contrast to the IEL population[23]. When disease,

such as Crohn's Disease (CD) or intestinal bowel disease, occurs DCs in the intestine migrate to lymphoid organs in response to the altered stimuli they encounter[24].

The mucosal immune system produces its own unique form of antibody, known as secretory IgA (sIgA). The production of IgA secreting B cells is initiated in the PP and mesenteric lymph nodes where IgM^+ B cells undergo Ig class switching from IgM to IgA[25]. IgA B cells then mature into plasma cells which localize within the LP and begin secreting IgA. IgA is bound by secretory component (SC) which is produced by epithelial cells and provides two functions[26]. First, it aids in the transport of sIgA across the epithelial barrier[27]. Secondly, it protects IgA from degradation by proteases in the lumen[28]. This is accomplished by protecting the Fc portion of the antibody and preventing access by proteases such as pepsin, papain and gastric acid.

Functionally, sIgA attacks bacteria and viruses by attaching to the surface molecules that these pathogens use to attach to mucosal epithelial cells[29]. sIgA can also attack toxins that are secreted by bacteria. This, of course, protects the GIT and allows pathogens to be expelled from the body in the mucus[30]. When the sIgA-antigen molecule is detected in the ileum it is taken up and transported to the liver. There, Kupffer cells destroy the antigen and allow the free sIgA to recirculate through bile back to the GIT[2]. It is interesting to note that the sIgA system does not fully develop in humans until age four[10].

Although the mesenteric lymph node (MLN) appears similar to other lymph nodes in the body they have very specific and unique functional properties. MLNs are located in the mesentery adjacent to most of the intestinal tract. Their locations follow the cranial mesenteric artery and begin roughly where the duodenum and jejunum meet. The MLNs continue as part of the mesenteric attachment from this point and extend distally to the rectum[31]. Upon antigenic

stimulation, DCs home to the draining lymph node and, along with lymph node stromal cells, release retinoic acid[32]. The presence of retinoic acid in the MLN causes T cells with the homing molecule integrin- $\alpha 4\beta 7$ and chemokine receptor CCR9 to migrate to the MLN[33]. T cells that home to the MLN favour Th2-type responses over Th1-type responses[34]. Along with T cells, the MLNs are also important in inducing B cell homing to the gut. MLNs also support the induction of foxp3-expressing T regulatory cells and IgA production. Furthermore, mice undergoing mesenteric lymphadectomy lose the ability to initiate oral tolerance[35]. Thus, the MLN performs many important functions relating to mucosal immunity.

1.2 Neonatal Immunity

During gestation the innate immune system develops and can provide protection against infection to fetal calves, although the acquired immune system is also developing at this time [36]. Fetal calves have the ability to respond to antigens but the response changes throughout gestation[37]. By the end of gestation all components of the acquired immune system are present, including the antibody response and innate immune defences, such as interferon and complement, are being produced [38, 39]. These components will not be fully active, however, until 2-4 weeks after birth [40]. During the last month of gestation T lymphocytes traffic away from the peripheral blood and begin to move into the lymphoid tissues. B lymphocytes increase from 1% during gestation to approximately 20% of PBMCs at 1-2 months of age [41]. Another study noted that the percentage of sIgM⁺CD21⁺ cells in blood was 6% in animals less than 1 week old and 33% in 7 week old animals [42]. The low number of B cells in blood during gestation correlates with a low number of antibodies [43]. At birth complement is at approximately 50% of adult levels [44]. Neutrophils produced by the fetus have reduced function

but this is remedied to some extent by neutrophils acquired through colostrum [45]. By the first week of life neutrophils are able to mount a proper response [46]. The DCs that are present in neonatal mice have limited ability to present antigen and activate the acquired immune system [47]. Proper immune responses are not always generated when the fetus encounters pathogens. For example, an infection with bovine viral diarrhea virus (BVDV) during the first half of gestation often fails to initiate protective immune response because it is recognized by the immune system as self and can lead to debilitating conditions throughout the calf's life [48-50].

At birth, B cells make up only a small percentage of total lymphocytes but gradually increase as the animals age [42, 46]. Newborn calves have only trace levels of endogenously produced antibodies in the first three days after birth and it isn't until day 4 when endogenous IgM is detected and days 16-32 when IgA and IgG are present [51, 52]. CD4, CD8, and $\gamma\delta$ T cells are present in the lymphoid tissues of newborns, such as the lymph nodes, but are in lower numbers than in adults [38]. The ratios between the different T cell subsets also changes slightly as calves age.

Because of the absence of microorganisms in the uterus, calves are born immunologically naïve. As they experience the environment they encounter antigens which initiate acquired immune responses. In the first few hours and days of life calves rely heavily on colostrum from their mothers to provide them with antibodies, cytokines and cells [53]. Colostrum is absorbed by the intestinal cells and endocytosis through transport vacuoles [54]. The ability to absorb colostrum is highest immediately following birth and begins to decrease by 6 hours post-partum and is completely absent by 48 hours after birth [55].

Maternal antibodies are an important component of colostrum and in calves IgG1 is the dominant antibody that is transferred to the newborn [56]. Cytokines are another important

component of colostrum. Cytokines that have been detected in colostrum include IL-1, IL-6, TNF- β , and IFN- γ [57]. It is not known if these cytokines are produced by leukocytes present in the colostrum or by the mammary gland itself. Cytokines received through colostrum can be detected in the bloodstream of newborn calves shortly after absorption [58]. There are also between 10^6 and 3×10^6 leukocytes/ml of colostrum. These leukocytes are comprised of macrophages, lymphocytes and neutrophils [59]. It is hypothesized that these leukocytes promote the development of APCs, which are very important in the development of the acquired immune response [40]. Lymphocytes from colostrum that are pathogen-specific have been detected at day 1 after birth but are absent at day 7 after birth [60].

During the fetal stages in mice chemical mediators such as prostaglandin E2, progesterone, and various cytokines are produced by the fetus and work to suppress Th1 responses and promote Th2 responses and antibody production [47, 61]. Breaking this apparent Th2 bias in the newborn has been the focus of much research in vaccine studies. Furthermore, maternal antibody interference is also a concern in the area of parenteral vaccine delivery to neonates. There has been much documentation of this occurring with modified-live vaccines injected parenterally for diseases such as BVDV [62], bovine respiratory syncytial virus (BRSV) [63, 64], bovine herpesvirus-1 (BHV-1) [65], *Mannheimia haemolytica* [66], and rotavirus [67]. Because of the interference of maternal antibodies it is very important for the vaccine to be delivered when the level of maternal antibodies is low enough for an active immune response to be generated by the calf itself. Unfortunately, the ideal time for vaccine delivery varies for each vaccine and is dependent on each animal and the rate of decay of the maternal antibodies, which is usually 16-28 days [68]. Methods have been developed to overcome the maternal antibody interference which revolve around changing the route of vaccine delivery and the use of

adjuvants. Using adjuvants with parenteral vaccines can help overcome the inhibition associated with maternal antibodies. This was shown for vaccines against BVDV [69, 70] and also against BRSV [71]. Mucosal delivery of modified-live viral vaccines to either the upper or lower respiratory tract has shown promise, possibly because of interferon induction of an anti-viral state in the body [72, 73]. Recent studies have confirmed, that mucosal delivery of modified-live viral vaccines to the upper respiratory tract of newborn calves is an effective strategy to avoid maternal antibody interference.

It is clear from recent studies that calves are born with the immune capacity to protect the body from immune diseases. However, studies of newborn calf immunity have been limited by the parenteral delivery of vaccines and maternal antibody interference but evidence is emerging that the mucosal immune system is capable of responding rapidly to foreign antigens. The mucosal immune system is well-equipped during gestation and early life to acquire and present antigens to the acquired immune system. This allows the immune system to fight infection and also to respond to vaccination challenges. Research into mucosal immunity allows us to better understand how to make more efficient vaccines.

1.3 Commensal Microflora

Mammals are born from a sterile environment but during birth begin encountering commensal microflora and pathogens that begin to colonize the GIT and shape the mucosal immune system [74]. There is increasing evidence that commensal microflora directly impacts development of the immune system located at mucosal surfaces. Commensal microflora can be found throughout the GIT and due to its geographical location the vast majority of these organisms are anaerobic in nature [75]. Adult humans have greater than 10^{10} organisms per gram

of GIT contents in the distal GIT, but only 10^4 organisms/gram of contents in the duodenum [76, 77]. The breakdown of fibre, which can provide ruminants with a major portion of their energy requirement, is actually performed by microorganisms in the rumen [78]. Each region of the GIT has its own distinct commensal microflora pattern, leading to even more diversity within the GIT [79, 80]. There are even differences between bacteria in the ingesta and those attached to the mucosa (Malmuthuge et al. manuscript in preparation).

Metabolically, the effect of commensal microflora in the GIT has been compared to that of the liver, which performs many functions in the body [81]. In ruminants, an estimated 40-60% of all dietary protein is digested by GIT microbes and the products are, in turn, used by the host [82]. Commensal microflora can synthesize important metabolites such as short-chain fatty acids (SCFA) and vitamin B12, which are used by the host [83]. Microbial fermentation in the rumen breaks down ingested material to volatile fatty acids (VFA) such as acetate, butyrate and propionate. VFAs then enter the bloodstream and can be used as an energy source by the body [82]. VFAs have also been known to inhibit the growth of pathogens such as *Salmonella* spp. in the GIT [84].

The use of germ-free (gnotobiotic) animals has demonstrated the important role commensal microflora plays in the development of the MALT in the intestine and other body organs. For example, gnotobiotic animals have thinner villi and, as a result, decreased intestinal mass, surface area and vasculature [85]. The heart, spleen, liver and adrenal glands do not grow to the same size in gnotobiotic animals as animals colonized by microorganisms [86]. Circulation is also reduced in gnotobiotic animals, which affects the animal's overall health [83]. Reintroducing commensal microflora also has a profound effect on DCs in the porcine jejunal LP. Prior to reintroducing *E. coli* spp. the lymphoid tissue of villi and crypts were devoid of

DCs. After the jejunum was repopulated with *E. coli* spp. extensive DC and T cell recruitment took place in the epithelium and LP[87].

In mice, the GALT, which includes the PP, LP and IEC, requires commensal microflora for normal development and function [88]. The PP in gnotobiotic mice is devoid of germinal centres, which are required for the generation of acquired immune responses. This leads to a decrease in IgA-secreting plasma cells in the LP [89]. $CD4^+$ and $CD8\alpha^+\alpha\beta TCR^+$ T cells are reduced in the absence of commensal microflora [90, 91]. Germ-free animals have difficulty in developing oral tolerance and develop atopic responses in the GIT [92-94]. Studies in germ-free pigs have shown that the distal small intestine is characterized by long villi populated by enterocytes containing large cytoplasmic vacuoles and a reduced LP, which contains fewer cells[95]. It has also been postulated that the interaction between TLRs and commensal microflora-derived molecules is responsible for many of the benefits they provide to us [83].

The impact of antibiotic usage also illustrates the need for commensal microflora. When humans are given broad-spectrum antibiotics, which removes commensal microflora from the intestine, the Gram-positive bacteria *C. difficile* can proliferate unchecked and cause illness [96]. It is thought that antibiotic usage eliminates, commensal microflora which compete against pathogens for nutrients and limit their replication.

1.4 Ileum and Jejunum

The length of the intestinal tract, from the duodenum to the rectum, is estimated to be between 33 and 59 metres in adult cattle [31]. Given this length, it is easy to understand how the area of the mucosal surfaces can be greater than $300m^2$. The small intestine is between 27 and 49 metres in length, while the large intestine is between 6 and 10 metres in length [31]. The

intestinal tract is divided into 6 major functional regions. In order from proximal to distal these regions are the duodenum, jejunum, ileum, cecum, colon and rectum (Figure 1.1). The main function of these regions is nutrient absorption but they also have a strong immunological component.

There are significant anatomical differences between the ileum and the jejunum. In cattle, these two regions can be defined anatomically in two distinct ways. First, the ileum is defined as the terminal region of the small intestine proximal to the beginning of the ileo-cecal fold. Alternatively, the ileal region has been defined by the presence of a continuous Peyer's patch (PP) while the jejunum is defined as the region within which discrete PPs are located [97]. It is possible to identify the ileum from the serosal surface, since the continuous PP causes a thickening of the intestinal wall and this tissue appears darker than the surrounding mucosa.

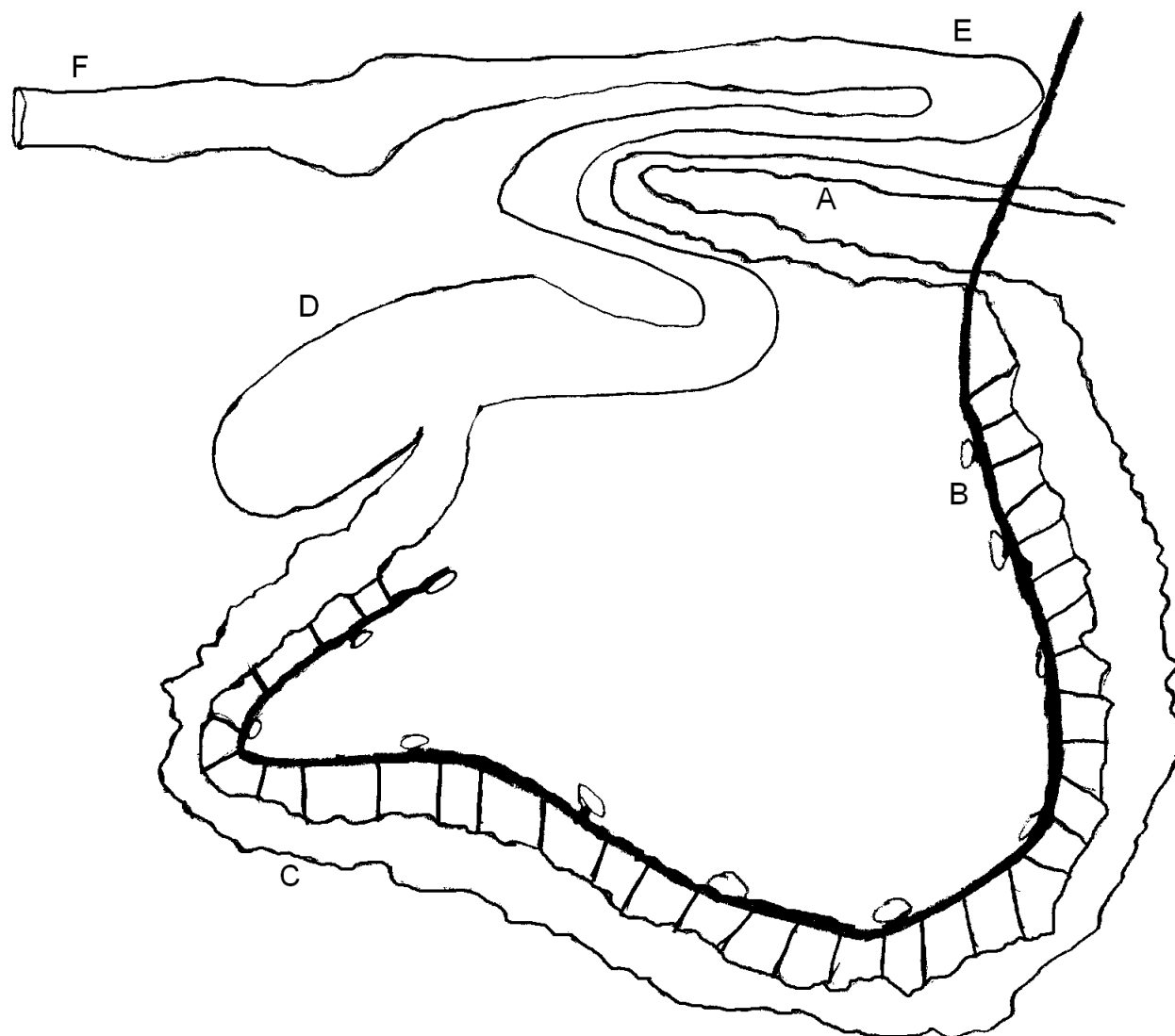


Figure 1.1. Anatomy of the intestine including lymph nodes and blood vessels. The small intestine begins at the distal end of the pyloric sphincter then continues on to the duodenum (A), jejunum (B) and ileum (C). The large intestine then begins at the cecum (D), and includes the colon (E) and rectum (F). The entire intestinal tract is 33 - 59 metres in length in adult cattle.[31]

The jejunum is the segment of small intestine, located between the duodenum and the ileum. The start of the jejunum is defined by the beginning of the mesenteric attachment and the absence of Brunner's glands, as is found in the duodenum. The jejunum contains PP that appear randomly every 15 - 30 cm. The human jejunum contains approximately 20 - 30 PP but this number may be as high as 70. The number of PP is highest at the time of birth where it is possible to find over 200 PP [98]. However, in germ-free pigs there are approximately 15 PP at birth and this number gradually increases as the animal ages [97]. In vivo studies have determined that the jejunal PP is a more efficient site for the induction of mucosal immune responses compared to the ileal PP [99].

The ileum is the last segment in the small intestine. It is bordered by the jejunum proximally and the cecum distally. The ileum terminates at the ileo-cecal valve, which provides access to the cecum. Metabolically, the role of the ileum is to absorb nutrients that have bypassed the duodenum and jejunum. It is also notable as a specific site for Vitamin B₁₂ uptake and bile acid reabsorption [100]. Physiologically, it appears similar to the jejunum, with the exception of the continuous PP in ruminants.

The ileum and jejunum also share a number of common features. Both contain thousands of villi, which serve to increase the mucosal surface area and the number of absorptive epithelium. This large mucosal surface allows more efficient nutrient uptake but this large interface between the body and the environment creates more opportunity for pathogen entry. Because of its length, the ileum and jejunum is extensively folded within the abdominal cavity but the mesentery anchors the intestine and provides blood supply, enervation, and lymphatic drainage through the cranial mesenteric artery [31]. Numerous MLN are situated in the mesenteric attachment along the length of the small intestine.

1.5 Mucosal Leukocyte Isolation

Numerous approaches have been used to isolate mucosal leukocytes from intestine. EDTA and collagenase digestion of intestinal tissue have been undertaken by a variety of researchers since the 1970's. Clancy (1976) used mechanical disassociation of intestinal tissue to analyze lymphocytes in patients with CD [101]. Subsequently, Bland et al (1979) compared mechanical and enzymatic digestion methods to release mucosal leukocytes from human GIT and concluded that higher yields and more active cells were released using enzymatic means [102]. Since then many groups have performed similar experiments in humans [103, 104], pigs [105, 106], and mice [107-110].

The first experiments involving bovine IEL and lamina propria leukocyte (LPL) isolations were performed by Nagi et al [111]. These experiments established the protocol for extracting mucosal leukocytes from bovine small intestine. They established the baseline values for lymphocytes in proximal jejunum and described the composition of IEL as 60% T cells and 10% B cells. LPL were characterized as 45% T cells and 28% B cells and there was no characterization of myeloid cells. Further experiments by this group investigated the *in vitro* effects of stimulating IEL and LPL with a variety of cytokines [112, 113] and phenotypically characterize the IEL, LPL and PP cells [114]. Again, no mention was made of either mucosal macrophages or DCs. Since the initial work of Nagi et al., bovine LPL have been analyzed for responses to foreign antigens and toxins and the effect of nutrition on IEL and LPL development has been investigated [115-117].

Phenotypic analysis of ileal IEL and LPL was completed by Wyatt et al [118] by comparing newborn, 1.5 week old and 3 week old calves. They concluded that CD3⁺ T cells in the IEL and LPL cellular compartments significantly increased in number with age when

comparing newborn to 1.5 and 3 week old calves. Furthermore, they noted that sIgM⁺ B cells increased in number with age, but this increase was not statistically significant. Similar results were seen in CD8⁺ and $\gamma\delta$ TcR T cells with these cell types increasing in number from birth to 3 weeks of age. Again, these studies did not include any analysis of either mucosal macrophages or DCs.

My studies build on those by Nagi et al by using their protocol to isolate a combined population of both IEL and LPL from the ileum and jejunum. Furthermore, my studies are the first to include an extensive analysis of mucosal myeloid cell populations and compare these mucosal macrophage and DC populations to the myeloid cell populations circulating in blood. This is an important comparison since immature myeloid cells are generated in the bone marrow, released into blood, and then recruited to tissues. A comparison of blood and tissue myeloid cells is critical when asking the question whether there is selective recruitment of myeloid cells or local differentiation of mucosal macrophages and DC subpopulations. Comparing these two populations provides a better understanding of immune system development in the small intestine.

CHAPTER 2

MUCOSAL DENDRITIC CELL DIVERSITY IN THE GASTROINTESTINAL TRACT

Fries, P.N. and P.J. Griebel, *Mucosal dendritic cell diversity in the gastrointestinal tract*. Cell Tissue Res, 2011 Jan; 343(1):33-41.

2.1 Abstract

The discovery of DCs in skin by Paul Langerhans in 1868 identified a cell type which has since been recognized as a key link between innate and adaptive immunity. DCs originate from bone marrow and disseminate through blood to all tissues in the body and distinct DC subpopulations have been identified in many different tissues. DC diversity is apparent throughout all mucosal surfaces of the body but the focus of this review article is DC diversity throughout the gastro-intestinal tract (GIT). DC subpopulations have been well characterized in the oral cavity and small intestine but DC characterization in other regions, such as the esophagus and stomach, is limited. Substantial research has focused on DC function during disease but understanding the regulation of inflammation and the induction of acquired immune responses requires combined phenotypic and functional characterization of individual DC subpopulations. Furthermore, little is known regarding mucosal DC subpopulations in the GIT of the neonate and how these DC populations change following colonization by commensal microflora. The current review will highlight mucosal DC diversity and discuss factors that may influence mucosal DC differentiation.

2.2 Introduction

In 1868, a German medical student named Paul Langerhans working in Berlin viewed, perhaps for the first time in history, a DC. He obtained human skin and stained tissue sections with Cohnheim's gold chloride stain[119]. Based on cellular morphology, he concluded these cells had a nervous function [2]. The cells he discovered would become known as Langerhans Cells (LCs) and were actually skin DCs. It would be nearly 100 years before another scientist would be able to build on Langerhan's observations when Birbeck granules were discovered in 1961 and in 1962 were proven to be unique to LCs[120, 121].

In 1973 Ralph Steinman and Zanvil Cohn discovered a new type of cell in the mouse spleen based on morphology and tissue distribution. Using a phase-contrast microscope Steinman observed cytoplasmic processes emanating from the cell and thus called it a DC[122]. Over the next few years this group continued to characterize DCs based on morphological and functional abilities and subsequently other attributes were used to define DCs as a distinct cell type[123]. Initially, it was thought DCs lacked pinocytotic ability[124], although this was later refuted[125]. An important discovery made in 1974 was that DCs were derived from precursor cells in the bone marrow[126]. Cells with properties similar to mature DCs were absent in bone marrow, which suggested DCs must originate from a bone marrow precursor cell. DCs were identified that exhibited responses similar to lymphoid cells and another significant step forward came in 1979 when splenic DCs were isolated and purified[127] which created the opportunity to perform phenotypic and functional studies. Subsequently, DC research rapidly expanded to include the analysis of DC phenotype and function in a variety of other organs and species.

2.3 DC Lineage

DCs are phenotypically and functionally diverse, which has complicated investigations of cell lineage[128]. Causing even more confusion is the use of phenotype and function, either separately or in combination, to define DC subsets and both these questions are important when investigating cell lineage. Once cell lineage is established it is then important to determine from where the cells originate and how cells are recruited to a specific tissue. Finally, it is important to determine if specific DC subpopulations are recruited to a tissue or if immature DCs (iDCs) are recruited and then differentiate under the influence of the local microenvironment.

Like all leukocytes, DCs are derived from a common hematopoietic stem cell (HSC). What makes DCs distinct from other leukocytes is their lineage commitment, which is determined by a number of growth factors. Expression of the hematopoietic growth factor receptor, Csf-1r, is important for the generation of most leukocytes [129]. However, the Flt3 receptor and Flt3 ligand (Flt3L) play an important role in the generation of both pre-DCs and plasmacytoid DCs (PDCs)[130]. In lymphoid tissues there are two known DC progenitor cells: monocyte/macrophage and DC precursor cells (MDP) and common DC precursor cells (CDP). Both these precursors have the ability to produce PDCs and conventional (c)DCs[131], however only MDP have the ability to produce monocyte/macrophages[132]. In the bone marrow CDPs give rise to preclassical DCs (pre-cDCs) and PDCs [133]. PDCs and pre-cDCs then traffic through the blood and are recruited to various tissues. PDCs differentiate from CDPs just as pre-cDCs do in the bone marrow and then traffic to target tissues [134]. Pre-DCs are also recruited to various tissues and then differentiate into a variety of phenotypically and functionally distinct DC subsets.

PDCs arise from HSCs in the bone marrow similar to all leukocytes and comprise 1-2% of all cells in that region[135]. PDCs are classed as DCs because they are very similar in function and development to other types of DCs. The PDCs are defined as distinct subpopulation based on an atypical DC morphology, recirculation and homing patterns, a prolonged survival time, and they share common properties with B cells[135]. PDCs are also unique in their capacity to respond to viral infections by secreting large amounts of type 1 interferon[136].

Murine mucosal DCs appear to arise from monocytes by first expressing Ly-6C⁺ and then traffic through the bloodstream and becoming CX3CR1⁺ LP DCs. Another possible pathway for LP DC development is by differentiating from pre-cDCs, arising from CDPs. These cells are thought to eventually become CD103⁺ LP DCs[134]. In cattle we have observed a variety of distinct DC subsets in the LP which are absent or rare in blood (Table 2.1). For example, CD11c^{hi}CD13⁺ are absent in the blood of calves but approximately 10% of ileal and jejunal LPLs are CD11c^{hi}CD13⁺ (Fries and Griebel, unpublished observations). These observations support the conclusion that iDCs are recruited to mucosal surfaces and differentiate into phenotypically distinct subsets under the influence of the local microenvironment.

Table 2.1. Monocyte and DC subpopulations present in the blood and small intestine of weaned calves (6 months old).

Phenotype	Blood	Intestine (jejunum)
CD11c ^{hi} MHC Class II ⁺	Present ¹	Present
CD11c ^{hi} MHC Class II ⁻	Rare ²	Rare
CD11c ^{hi} CD11b ⁺	Present	Present
CD11c ^{hi} CD11b ⁻	Minor ³	Present
CD11c ^{hi} CD13 ⁺	Absent	Present
CD11c ^{hi} CD13 ⁻	Present	Rare
CD11c ^{hi} CD14 ⁺	Present	Minor
CD11c ^{hi} CD14 ⁻	Present	Present
CD11c ^{hi} CD26 ⁺	Minor	Present
CD11c ^{hi} CD26 ⁻	Present	Present
CD11c ^{hi} CD205 ⁺	Present	Present
CD11c ^{hi} CD205 ⁻	Present	Present

1. Present = 10-90%; 2. Rare = 0-2%; 3. Minor = 2-10%

2.4 DC Function

DCs are an important component of the innate immune system and are found throughout the body. The main function of DCs is to interact with pathogens and present their antigens to cells of the adaptive immune system, such as T cells and B cells[21]. This initiates effector responses to enhance pathogen clearance and establish immune memory specific to individual pathogens. For this reason it is said that DCs are the bridge between the innate and adaptive immune system. DCs also have the ability to secrete cytokines depending on their location in the body and their state of differentiation and activation. For example, PDCs have potent anti-viral effects because of their ability to secrete type 1 interferon, whereas myeloid DCs secrete vast amount of IL-12 in response to antigenic stimulation[136, 137]. Based on location, DCs can be divided into two broad categories: blood DCs and tissue DCs. Blood DCs circulate in the bloodstream searching for antigen and have been extensively studied because of their ease of isolation. Tissue DCs reside throughout the body, including mucosal surfaces, and DC populations are specialized for the tissue in which they reside.

2.5 Mucosal DC Diversity

The surface molecules used to define DC phenotype vary among species. Some molecules have been conserved across species and provide a basis for interspecies comparison. For example, CD11b and CD11c molecules are found on murine, human and bovine DCs (Table 2.1). In contrast, some molecules are species-specific and no functional orthologue may be known. Despite these limitations it has been possible, within individual species, to begin appreciating the diversity of DC subsets identified at specific mucosal surfaces.

Mucosal surfaces can be divided into two distinct functional groups: type I and type II mucosa. Mucosal surfaces covered by simple columnar epithelium are classified as type I mucosa and include the small and large intestine, the respiratory system, and the upper female reproductive system (oviduct, ovary, uterus, endocervix). Mucosal surfaces covered by noncornified, stratified epithelial layer are classified as type II mucosa and include the mouth, esophagus, cornea and the lower female reproductive system (ectocervix, vagina) [21]. DC access to surface antigens varies between these two types of mucosal surfaces and there is emerging evidence that mucosal DC subpopulations also vary among mucosal sites. Understanding the functional and phenotypic differences in mucosal DC subsets is important for understanding the immune regulatory roles DCs play through interactions with commensal microflora, pathogens, and food antigens.

2.6 Oral Cavity

The oral cavity is the first site of exposure to many pathogens and oral mucosal DCs (omDCs) are present throughout the mouth. Organized lymphoid tissue is also present in the oral cavity, including the tongue and tonsils, and function as important immune induction sites. Investigation of omDCs has increased with interest in developing sublingual immunotherapy (SLIT), whereby antigen-specific tolerance can be induced by administering antigen under the tongue, a region rich in DCs. In mice PDCs ($B220^{+}120G8^{+}$), myeloid DCs ($CD11b^{+}CD11c^{+/-}$), and LCs ($CD207^{+}$) [Table 2.2] have been found in the oral submucosa, the submucosa/mucosa interface, and the mucosa, respectively[138]. The function of omDCs is to ingest, process, and present antigen to $CD4^{+}$ T cells, where they will stimulate the production of either $IFN\gamma$ or IL-10.

The production of IL-10 is linked to immune tolerance and the induction of Th1 regulatory T cell responses.

Table 2.2. Mouse mucosal DC subsets throughout the GIT

Tissue	Subsets	Localization	Reference
Oral Cavity (Mucosa)	PDCs (B220 ⁺ 120G8 ⁺)	Submucosa	[138]
	Myeloid (CD11b ⁺ CD11c ⁺)		[138]
	LCs (CD207 ⁺)	Mucosa	[138]
Esophagus	Not described		
Stomach	Not described		
Small Intestine	CD11c ^{hi} CX3CR1 ⁻ CD11b ^{hi/lo} CD103 ⁺	LP	[139]
	CD11c ^{hi/lo} CX3CR1 ⁺ CD70 ⁺ CD11b ^{hi} CD103 ⁻	LP	[140]
Large Intestine	CD40 ^{lo} MHC Class II ^{lo} CD80 ⁺ CD86 ⁺	LP	[141]

Phenotype studies of DCs in the human tongue have identified distinct DC subsets which reside in the fungiform papillae (FP) of the tongue. CD11c⁺ myeloid DCs and DC-SIGN⁺ immature DCs are both found in the epithelium and LP, but mainly in the LP [Table 2.3]. CD83⁺ mature DCs are found in both the epithelium and LP, while CD1a⁺ LC DCs are found solely in the epithelium[142]. The study of DCs and immune cells in the tongue may also have relevance due to their possible contribution to taste dysfunction in patients who suffer from this disease.

Table 2.3. Human mucosal DC subsets throughout the GIT

Tissue	Subsets	Localization	Reference
Oral Cavity (Tongue)	Immature DCs (CD11c ⁺ DC-SIGN ⁺)	epithelium &	[142]
	Mature DCs (CD83 ⁺)	LP	
	LCs (CD1a ⁺)	epithelium & LP	[142]
Esophagus	CD83 ⁺ DC-SIGN ⁺	LP	[143]
Stomach	Immature (CD54 ⁺ CD80 ⁺ CD86 ⁺)	LP	[144]
Small Intestine	CD11c ⁺ CD83 ⁺ DC-SIGN ⁺ CCR6 ⁺ CCR7 ⁻ TLR-4 ⁺ TNF α ⁺	Subepithelial dome	[145]
Large Intestine	CD83 ⁺ CD86 ⁺ CD80 ⁻ CD25 ⁻	LP	[146]
	CD83 ⁺ and DC-SIGN ⁺	LP	[147]

Over the past 10 years research on LCs in the oral mucosa has increased greatly. Oral DCs were discovered to express high levels of high affinity receptor for IgE (FCεRI)[148]. FCεRI is a receptor on the surface of DCs, among other cell types, responsible for regulating allergic response[149]. Oral LC DCs differ from epidermal LC DCs in their expression of FCεRI, which could alter their involvement in allergic response[150]. On the other hand, nasal mucosal DCs and oral mucosal DCs both share certain properties, such as a high expression of FCεRI, myeloid markers and costimulatory expression, but display differences when it comes to lineage, phenotype, and function[151]. Therefore, regional differences in DC phenotype and function are apparent within the oral cavity. Studies investigating age-dependent and species-specific differences among omDC subsets have not yet been undertaken. Studies in fetal sheep, however, confirmed DCs were present in the oral mucosa as early as the second trimester of pregnancy and effective antigen presentation could occur following exposure to oral antigens[152].

2.7 Esophagus

Esophageal DCs are poorly characterized but it is known that DCs inhabit the LP. DC phenotype in this and other mucosal compartments of the esophagus have, however, been limited to investigations of human disease states, such as Barrett's esophagus. This disease is characterized by chronic inflammation and mucosal epithelial dysplasia following chronic gastric acid reflux [153]. DC-SIGN and CD83⁺ DCs increase in density when comparing adenocarcinoma versus benign Barrett's esophagus[143] and DC infiltrating the esophagus during disease state have typical morphological features characteristic of DC[154] (Table 2.3).

Studies of DCs in healthy esophagus and comprehensive phenotypic and functional studies are lacking (Table 2.2).

2.8 Stomach

Similar to the esophagus, the analysis of gastric mucosal DCs has been performed primarily within the context of disease. Much of this research has focused on DC responses to *Helicobacter pylori* (*H. pylori*) in the gastroduodenal region. It has been reported that the healthy gastric mucosa of mice is devoid of DCs[144] (Table 2.2). However, a study of the rumen and fore stomach of adult and fetal sheep concluded that LCs were present[155]. DCs are reported to be recruited to the gastric mucosa within 6 hours after primary *H. pylori* infection[156]. The main site of infiltration for DCs is the LP of the gastric mucosa[157] and recruited DCs express costimulatory markers such as CD54, CD80 and CD86 [144] (Table 2.3). Overall, DCs are thought to contribute to chronic inflammation during *H. pylori* infection through the induction of Th1 biased responses[158]. The mechanisms by which DCs contribute to chronic inflammation in *H. pylori* are not completely understood. One theory is that the bacteria replicate in the autophagosome of DCs, impair DC function and induce autophagy[159]. This internal replication also causes the LC3, LAMP1 and MHC Class II molecules to be held within the vacuole and as a result MHC Class II molecules cannot be expressed on the surface of the cell.

2.9 Small Intestine

Although the ileum and jejunum have physiological and functional differences the DC subpopulations are often investigated within the small intestine without identifying the specific location[99]. The most notable immunological difference between ileum and jejunum is the

structure of the organized lymphoid tissue known as PP. In ruminants, pigs and many mammalian species the ileal PP consists of a continuous aggregate of lymphoid follicles while the jejunal PP consist of multiple discrete lymphoid aggregate [160]. DCs are located in the LP throughout the small intestine, though due to the close proximity of LP and mucosal epithelium it is difficult to discriminate between intraepithelial DCs and DCs present in the LP [161].

Approximately 10-15% of LP leukocytes (LPL) in the murine small intestine are thought to be DCs [162]. CD11c⁺MHC Class II⁺ DCs make up approximately 20% of all CD45⁺ LPL isolated from the ileum and jejunum of 3 week old calves. There are, however, age-related decreases in DC frequency and 5% of ileal and 10% of jejunal LPL are CD11c⁺ MHC Class II⁺ DCs in weaned calves (Fries and Griebel, unpublished observations).

Murine DCs in the small intestine are typically categorized into two groups: CD103⁺ and CD103⁻ (Table II). CD103 is integrin α_E and is expressed by 70% of CD11c^{hi} DCs in the murine small intestine LP [139, 163]. CD11c^{hi}CD11b^{hi/lo}CD103⁺ DCs are thought to promote differentiation of Foxp3⁺ Tregs [162]. These cells are also CX3CR1⁻ indicating they are unable to extend dendrites into the intestinal lumen since this chemokine receptor has been associated with dendrite formation [164]. Despite a lack of transmucosal dendrites and a known role in immune tolerance the CD103⁺ DCs can induce expression of homing markers CCR9 and $\alpha 4\beta 7$ on T Cells. Another group of DCs identified in the LP are CD11c^{hi/lo}CX3CR1⁺CD70⁺CD11b^{hi}CD103⁻ and this DC subset has been implicated in Th17 differentiation from naive T cells [140]. It is also believed that CD103⁻ DCs perform innate immune function, such as chemokine-mediated attraction and antigen clearance, which CD103⁺ cells cannot perform [165]. All DCs found in the interfollicular region of the PP also express CCR7, which regulates chemotaxis and survival in mature DCs [166]. There is research that

indicates that the unique functional properties of CD103⁺ DCs are conserved between humans and mice [167] but there is no information if this DC subset marker is conserved among other species.

Research in healthy human small intestine is very limited but investigations into CD provide some insight into the DC populations present in the ileum. In CD there is an accumulation of CD11c⁺CD83⁺DC-SIGN⁺ DCs in the subepithelial dome [145]. The accumulation of DCs in this region could be a result of the lack of the lymph node migratory receptor CCR7 and the high bacterial load that is found in this region [168]. These DCs also increase from 0% to 1.6% when comparing healthy ileal wall and CD ileal wall, respectively [145]. Elevated TLR 4 expression and increased TNF α production may contribute to the inflammatory symptoms that accompany CD. TLR 4 is the receptor on myeloid DCs that recognizes lipopolysaccharide [169] and myeloid DCs isolated from the colon of inflammatory bowel disease patients have increased expression of CD83, TLR 4 and TNF α which may contribute to inflammation [24, 170].

Research into small intestine DCs of other species is very limited. We are currently analyzing DC diversity in the ileum and jejunum of young calves and age-related changes that occur in mucosal DC subsets. Our research also included parallel comparisons of DC/myeloid subsets in blood and the small intestine. This comparison provides information about both leukocyte trafficking and the local differentiation of mucosal DCs. We identified diverse DC subsets located with the LP of small intestine (Table 2.1). A significant difference in DC subsets was observed when comparing LPL isolate from the ileum and jejunum of 3 week old calves. CD11c^{hi}CD14⁺ DCs were significantly more abundant in the LP of the ileum (6.4%) than the jejunum (2.8%). This regional difference in DC subsets was no longer apparent, however, when

comparing DCs isolated from the intestine of weaned calves (Fries and Griebel, unpublished observations).

As mammals age marked changes occur within the immune system [171, 172]. These age-related changes are also apparent within the LPL of cattle. At 3 weeks of age CD3⁺ T cells account for approximately 60% of LPL and DCs account for nearly 20% LPL isolated from both the ileum and jejunum. At 6 months of age, however, CD3⁺ T cells have increased to 88% of ileal and 77% of jejunal LP. In contrast, DCs have decreased to 5% of ileal and 9% of jejunal LPL. Furthermore, specific DC subpopulations also change with age. In the ileal LP CD11c⁺CD13⁺, CD11c⁺CD26⁺, and CD11c⁺CD205⁺ DCs become more abundant with age but CD11c⁺CD14⁺ and CD11c⁺CD172a⁺ decrease in frequency between 3 weeks and 6 months of age. Similarly in the jejunum LP CD11c⁺CD13⁺, CD11c⁺CD26⁺, and CD11c⁺CD205⁺ DCs become more abundant at 6 months of age (Fries and Griebel, unpublished observations). These observations suggest that developmental differences in mucosal DC subsets may be present at birth but with exposure to commensal microflora there are substantial changes in mucosal DC subsets.

The interactions between the intestinal epithelial cells (IEC) and DCs are very important for maintaining homeostasis in the intestine and are accomplished through a number of different methods. IEC are the barrier that prevents pathogens from further entering the body. Microorganisms are sampled by IEC and are passed to DCs in the LP and SED through the use of transcytosis[173]. CX3CR1⁺ DCs are also able to sample microorganisms in the lumen by extending their dendrites between the tight junctions of the IEC[174]. Specialized IEC known as M cells can also deliver commensal microflora to LP and PP DCs. IEC and DCs also use cytokines to communicate. IEC express thymic stromal lymphopoietin, which limits IL-12

production by DCs, which inhibits Th1 induction. In vitro assays with monocyte derived DCs have shown that DCs are conditioned by IEC through ‘cross-talk’ and if this is lost DCs could lose the ability to secrete cytokines and influence immune responses [175]. The failure of intestinal DCs to induce an inflammatory response to commensal microflora is not well understood but one possibility is that anti-inflammatory molecules derived from IEC prevent DCs from initiating such a response [176]. This symbiosis between IEC and mucosal DCs is thought to be critical for maintaining the health of the intestine while inhabited by a diverse community of commensal bacteria.

There have also been studies investigating the role of TLRs on the surface of LP DCs. Murine LP DCs have higher levels of TLRs 2, 3, 4, 5, and 9 expression than DCs located in spleen and mesenteric lymph node and TLR expression level is lower in DCs located in the proximal small intestine versus the distal small intestine [177]. It has also been reported that CD11c^{hi}CD11b^{hi} LP DCs express high levels of TLR 5. When these cells are stimulated with flagellin they are able to induce B cells to differentiate into IgA producing plasma cells and also promote the differentiation of IL-17 Th1 cells [178]. TLR signaling also has the ability to down-regulate intestinal inflammation and is important for maintaining intestinal homeostasis [83].

2.10 Colon

Similar to many other regions of the GIT the colon LP contains diverse DC subsets[179]. Most research on the colon involves disease states such as inflammatory bowel disease and ulcerative colitis but there is some data available for the healthy murine colon (Table 2.2). The colon LP of healthy mice contains CD40^{lo}MHC Class II^{lo}CD80⁺CD86⁺ DCs in close association with the basement membrane of the villi [141]. These cells also exhibit high endocytic activity,

which suggests they are immature DCs. There are very few PDCs in colonic LP [180]. IL2^{-/-} knock-out mice develop spontaneous colitis and it was observed that CD40^{lo}MHC Class II^{lo}CD80⁺CD86⁺ DCs increased significantly in the colon LP following colitis. A similar increase in CD40^{lo}MHC Class II^{lo}CD80⁺CD86⁺ DCs was observed in the colon of severe combined immunodeficiency (SCID) mice [181, 182]. CD103⁺integrin $\alpha_v\beta_8$ ⁺ DCs are important for the suppression of colitogenic T cells [183, 184] and mice lacking this DC subset have fewer Tregs in the colon and are predisposed to develop colitis. DCs in the murine colon have low expression of TLRs 4, 5, and 9, while in humans colonic DCs have low expression of TLRs 2 and 4 [147, 170, 180]. However, in mice that suffer from CD or ulcerative colitis there is upregulation of TLRs 2 and 4 on colonic DCs. A high expression of TLRs 2 and 4 may affect microbial recognition and exacerbate the cycle of intestinal inflammation. Whereas, low level TLRs 2 and 4 expression in healthy colon may be protective and prevent excessive interaction with commensal microflora [170]. Increased TLRs 2 and 4 expression is also seen on IEC in CD and ulcerative colitis patients [185].

Research on human colonic DCs has focused mostly on ulcerative colitis patients (Table III). Limited description of DCs in healthy human colon has revealed that these cells display an immature phenotype and are CD83⁺CD86⁺CD80⁻CD25⁻ [146]. Human colonic DCs also express CD83⁺ and DC-SIGN⁺[147]. In ulcerative colitis patients DCs accumulate in the colon [186]. DCs isolated from ulcerative colitis patients are CD83⁻CD80⁺DC-SIGN⁺, produce IL-12 and IL-18, and promote Th1 development [147]. A higher number of CD86⁺CD40⁺ DCs are found in the blood and colon LP of patients with ulcerative colitis [186]. There have been few descriptions of colonic DCs in other species but it is assumed that they are present.

2.11 Peyer's Patches

Peyer's patches are prominent MALT located in the small intestine and play an important role in the induction of IgA antibody responses. The structure and function of PPs have been studied extensively and several well defined immune compartments have been identified. Follicle-associated epithelium (FAE) provides a direct interface with the luminal contents and transcytosis of material to the underlying subepithelial dome region and lymphoid follicles. The lymphoid follicles contain primarily B cells and are interspersed among the interfollicular (IF) lymphoid tissue which is rich in T cells and function as sites of T cell trafficking. These structurally defined regions within the MALT perform immunologically distinct functions and are characterized by phenotypically distinct DC subpopulations.

Immature DCs are associated within the FAE of mouse PPs and have been characterized as CD11b⁻ CD8a⁻ B220⁻ [187]. In contrast, the subepithelial dome region of the follicle contains a mixed population CD11b⁻ CD8a⁻ B220⁻ and CD11b⁺ CD8a⁻ B220⁻ DCs [188]. The IF region is unique in that it contains not only CD11b⁺ CD8a⁺ but also PDCs [189, 190]. Finally, the lymphoid follicles contain both non-hematopoietically derived follicular DCs (FDCs) and hematopoietically derived DCs [191]. Thus, within murine PPs there are specific patterns of DC localization but it is not known to what extent these restricted DC subset distribution patterns reflect DC migration and differentiation within each immune compartment.

Human PPs have been studied much less but CD11C⁺ MHC Class II⁺ DCs have been identified throughout all immune compartments. There is some evidence for selective DC distribution with CD11C⁻ MHC Class II⁺ DC-SIGN⁺ cells restricted to the sub-epithelial dome region of human PPs [192]. Studies of porcine PP also provide evidence for distinct DC subset distribution patterns. Immunohistochemical (IHC) analysis of jejunal PP revealed CD11b⁻

CD172a⁺ DCs were located in the subepithelial dome and CD11b⁻CD172a⁻ DCs predominated in the IF region[193]. Analysis of sheep PP confirmed MgATPase⁺ DCs were present within lymphoid follicles of the fetal ileal PP[194] and in young sheep MgATPase⁺ DCs were identified within the FAE, subepithelial dome region and follicles [195]. Neither flow cytometric nor IHC studies have been performed in sheep to determine if DC subsets are differentially distributed either within PPs or among MALT located throughout the GIT.

2.12 Conclusions

DCs have been recognized as key cells in the integration of innate and adaptive immunity and it is hypothesized that the differential capacity of individual DC subsets to recognize and respond to pathogen-associated molecular patterns (PAMPs) directly influences the induction of protective immune responses. This hypothesis is supported by the analysis of human plasmacytoid and myeloid DCs isolated from blood[196]. The analysis of TLR expression on gastro-intestinal DCs is limited but both the expression and responses to TLR ligands was markedly restricted in mucosal DCs[177]. This same investigation also revealed significant differences in TLR expression when comparing DCs isolated from different regions of the GIT. This is consistent with phenotype analyses which reveal specific distribution patterns for mucosal DC subsets throughout the GIT of many different species. As the number of surface markers used to identify individual DC subsets increase in complexity there is a confounding number of possible DC subsets[197]. It has been a challenge, however, to match DC phenotype characterization with a thorough analysis of DC function. Therefore, it is not known to what extent each DC subset possesses unique functional capacities in terms of PAMP recognition or T cell regulation. Furthermore, it is not known if individual mucosal DC subsets represent

immature or mature DCs that are differentiating under the influence of factors unique to each mucosal microenvironment.

Limited functional studies have revealed that individual mucosal DC subsets may possess unique cytokine secretion patterns or functional capacities in terms of T cell activation. Many studies performed with *in vitro* derived DCs have been used to define DC function[198-200] but it is difficult to extrapolate this information to the myriad of diverse DC subsets identified throughout mucosal tissues. The isolation of splenic DCs opened a new era in the investigation of DC biology. A major challenge that must now be resolved for further investigation of mucosal DC biology is the isolation and purification of sufficient numbers of tissue-derived DCs to perform function assays. These studies are critical to determine if individual DC subsets represent functionally unique populations with a restricted capacity to influence innate and adaptive immune responses. Understanding the link between DC phenotype and function will provide much greater insight when studying DC responses during disease and when using DCs as immune therapy.

The current review of mucosal DC subsets and their distribution throughout the GIT has revealed that little is known for many species. The limited information available for humans and other species supports the observation made in mice that there is a great diversity of mucosal DC subsets with marked regional differences in the tissue distribution of individual DC subsets. Interspecies comparisons are difficult since a wide variety of non-homologous surface proteins are often used to identify individual DC subsets. Mucosal DC diversity, however, raises intriguing questions regarding the link between DC activation and differentiation and the role of the local mucosal microenvironment in this process. Furthermore, in view of the key role DCs play in immune regulation it is critical to further investigate the function of individual mucosal

DC subpopulations. This will be a major challenge since access to mucosal tissues is often limited and isolating relatively rare mucosal DC subsets presents major technical challenges. Animal models, such as pigs or cattle, which provide access to blood, lymph, and mucosal tissue may provide sufficient DCs to perform function assays and study DC function in a variety of disease models. To explore the full potential of these alternative animal models, however, it will be important to identify monoclonal antibodies and other reagents to select the diverse array of DC subsets identified in the mouse model.

CHAPTER 3

RESEARCH OBJECTIVES AND HYPOTHESES

The intestinal LP is a very important mucosal immune compartment. It houses a wide variety of leukocytes which perform diverse immune functions. Understanding the cellular composition of this compartment and how leukocyte populations change with age will help to better understand the mucosal immune system in the small intestine. This was accomplished in the present investigation through the use of flow cytometry, IHC, cytopins, and cell sorting using flow cytometry.

The ileum and jejunum share many common anatomical features and, physiologically, appear quite similar. However, many differences have been discovered in the phenotype and distribution of mucosal leukocytes when comparing different regions of the GIT in other species. Thus, I hypothesize that the small intestine will be populated by diverse leukocyte populations within the LP and significant regional differences exist in the leukocyte subpopulations present in the ileum and jejunum of newborn calves (3 – 5 weeks old) and weaned calves (6 months old).

The immune system is constantly evolving as an animal ages and exposure to commensal microflora substantially alters the immune system in the small intestine. For these reasons I hypothesize that there will be substantial changes in the phenotype and distribution of leukocytes in the ileum and jejunum when comparing newborn and weaned calves.

CHAPTER 4

MUCOSAL DENDRITIC CELL SUBPOPULATIONS IN THE SMALL INTESTINE OF NEWBORN CALVES

Fries, P., Y.I. Popowych, L.L. Guan, T. Beskorwayne, A. Potter, L. Babiuk, and P.J. Griebel, *Mucosal dendritic cell subpopulations in the small intestine of newborn calves*. Dev Comp Immunol, 2011 Oct; 35(10):1040-51.

4.1 Abstract

Mucosal DC development in the newborn is poorly understood despite evidence that distinct DC subpopulations populate individual mucosal surfaces. Therefore, we investigated DC phenotype and distribution in the small intestine of newborn calves. DC phenotype was analyzed using flow cytometry and DC distribution was investigated with IHC. Purification of CD11c^{Hi}MHC Class II⁺ cells confirmed CD11c defined myeloid cells and a comparison of neonatal blood and intestine revealed distinct mucosal DC subpopulations. CD11c^{Hi}CD14⁺ cells were significantly more abundant in newborn ileum versus jejunum and CD335⁺ NK cells were the only lymphoid population significantly different in ileum versus jejunum. IHC revealed unique patterns of myeloid cell distribution within the mucosal epithelium, LP, and submucosa. CD11c⁺ cells were present within the jejunal but absent from the ileal intraepithelial compartment. In contrast, CD11b⁺ cells were present within the ileal but absent from the jejunal intraepithelial compartment. In conclusion, the neonatal small intestine is populated by diverse myeloid subpopulations and significant differences in regional distribution are established early

in life. These observations may have significant implications for the response of the newborn to both commensal microflora and enteric pathogens.

4.2 Introduction

Dendritic cells are present at all mucosal surfaces throughout the body[21]. Their role at these sites is to initiate innate immune responses, acquire foreign antigens, and present antigen in MALT. Distinct DC subpopulations have been identified at individual mucosal sites and the distribution of DC subpopulations varies among mucosal immune compartments [201]. The majority of studies characterizing DCs have been conducted with adult mice or humans and very little is known about the phenotype or distribution of mucosal DCs in neonates.

Numerous studies have analyzed DC phenotype and function in the small intestine of mice. Two distinct DC subpopulations have been identified in the LP of the murine small intestine: $CD11c^{Hi}CX3CR1^{-}CD11b^{Hi/Lo}CD103^{+}$ DCs and $CD11c^{Hi}CX3CR1^{-}CD11b^{Hi/Lo}CD103^{-}$ DCs, of which the former comprises 70% of mucosal DCs in the LP of the small intestine [139, 163]. The $CD103^{+}$ (integrin α_E) DCs play a role in promoting $Foxp3^{+}$ Treg differentiation [162] but lack the ability to extend transmucosal dendrites through the mucosal epithelium [164]. There are few studies characterizing DC subpopulations in healthy human intestinal tissue but mucosal DCs have been analyzed in disease states, such as CD. CD is associated with an accumulation of $CD11c^{+}CD83^{+}DC-SIGN^{+}$ DCs in the PP subepithelial dome region of the small intestine but these DCs are absent in healthy tissue [145]. Little is known, however, about mucosal DC development in the neonate of any species. There is evidence that the oral mucosa is populated with DCs during fetal development in sheep and exposure to a foreign antigen can effectively induce an acquired immune response [202].

Bovine mucosal DCs have not been characterized and extrapolation of information from humans or mice may be confounded by physiological differences between monogastrics and ruminants. DCs in a variety of bovine tissues have, however, been characterized and there is considerable variation in the phenotypes reported. CD11a⁺CD11c⁺CD13⁺CD172a⁺CD205⁺ immature DCs have been reported in the spleen of healthy calves [203, 204] and splenic DCs were further defined as MHC II⁺CD14⁻ as compared to macrophages which expressed CD14. DC populations in the thymic medulla have been described as CD11c⁺CD172a⁺ as well as CD1⁺CD172a⁺ [205]. The analysis of veiled cells isolated from afferent lymph has provided the most detailed phenotypic characterization of bovine DCs. Two distinct DC subpopulations were identified as MHC Class II⁺CD11a⁺CD13⁺CD26⁺ and MHC Class II⁺CD172a⁺ but lacking CD13 and CD26 [206, 207]. These observations have been confirmed and extended by demonstrating that CD205 is also expressed on bovine DCs in afferent lymph [208, 209]. In contrast, immature DCs isolated from blood were defined as CD11c⁺MHC Class II⁺CD14⁻ and morphologically they appeared veiled [210]. Thus, distinct DC subpopulations have been identified in variety of bovine tissues with CD13, CD26, and CD205 providing lineage specific markers when co-staining with MHC Class II or CD11c.

There are many functional and structural similarities among different regions of the bovine gastrointestinal tract (GIT). Common features include exposure of the mucosa to commensal microflora, a simple columnar mucosal epithelium, the LP is populated by an abundance of leukocytes, and an outer muscle layer provides peristalsis [211]. The ileal and jejunal regions in young calves, however, contain distinct MALT. The PP in the bovine ileum consists of a continuous aggregate of lymphoid follicles which function as a primary lymphoid organ for antigen-independent generation of the B cell immunoglobulin (Ig) repertoire [212]. In

contrast, the jejunal PP consists of multiple, discrete lymphoid aggregates which function as mucosal immune induction sites for the generation of IgA plasma cells [160]. Ruminant PP develop *in utero* and fetal immunization experiments have confirmed that the mucosal immune system can respond to foreign antigens which suggests that effective antigen-presentation can occur at this time [202]. Since DCs play a key role in linking innate and adaptive immunity we investigated both the phenotype and distribution of mucosal DCs in the small intestine of newborn calves.

4.3 Materials & Methods

4.3.1 Animals

Castrated male Holstein calves, between 7 to 10 days of age, were purchased from a commercial dairy. The calves were individually housed in the VIDO Animal Care facility and fed a diet of whole milk for a period of two to four weeks prior to tissue collection. During this period calves were examined daily by a veterinarian for body temperature and clinical signs of respiratory or enteric disease. Calves that remained clinically normal (n = 8) were humanely euthanized with an intravenous injection of Euthanyl[®] (240mg/ml; Bimeda-MTC Animal Health Inc., Cambridge, ON) at 21 days (n = 4), 28 days (n = 3), and 35 days (n = 1) of age. Experiments were conducted according to the Guide to the Care and Use of Experimental Animals, provided by the Canadian Council on Animal Care. All experimental protocols were approved by University of Saskatchewan Animal Care Committee.

4.3.2 Tissue collection

Intestinal tissue was collected within 10 minutes of euthanasia and a 10cm segment of ileum and a 15cm segment of jejunum were collected. Ileal tissue was collected immediately proximal to the ileo-cecal fold and jejunal tissue was collected 3-4 m anterior to the ileum and between two discreet PPs (Figure 4.1). Tissues were placed into ice-cold calcium and magnesium free Hanks Balanced Saline Solution (CMF-HBSS) supplemented with 5% fetal bovine serum (FBS; SeraCare, Milford, MA) and 1X antibiotic/antimycotic (100U/ml penicillin, 100µg/ml streptomycin and 0.25ug/ml amphotericin B; Life Technologies, Carlsbad, CA). Tissues were transported to the lab on ice.

4.3.3. Tissue digestion

Intestinal segments were opened by making an incision along the mesenteric attachment. Ingesta was removed and the mucosal surface washed with CMF-HBSS. Each intestinal section was placed in a petri dish (VWR, West Chester, PA) containing magnesium and calcium-free phosphate buffered saline (PBSA), cut into 1cm² pieces and transferred to a glass beaker supplemented with 5% FBS and antibiotic/antimycotic.

4.3.4. Removal of mucosal epithelium

Dithiothreitol (DTT, 10mM, Sigma-Aldrich, St. Louis, MO) was added to the CMF-HBSS and tissues were incubated for 15 minutes at 37° C on a shaker platform (Innova 4900, New Brunswick Scientific, Edison, NJ) at 150 revolutions per minute (RPM). Tissues were then transferred to beakers containing CMF-HBSS, 5% FBS, 1X antibiotic/antimycotic, and 10mM EDTA (EMD Chemicals, Gibbstown, NJ) and incubated at 37° C for 30 minutes while shaking at 150 RPM. This incubation was repeated a second time after transferring tissues to fresh

medium and the supernatant was again discarded. Intestinal fragments were then transferred to new beakers containing CMF-HBSS, 5% FBS, 1X antibiotic/antimycotic, 30 µg/ml DNase I (Sigma-Aldrich), and 200U/ml collagenase IV (Worthington Biochemical, Lakewood, NJ) and incubated for 60 minutes at 37° C with shaking at 150 RPM.

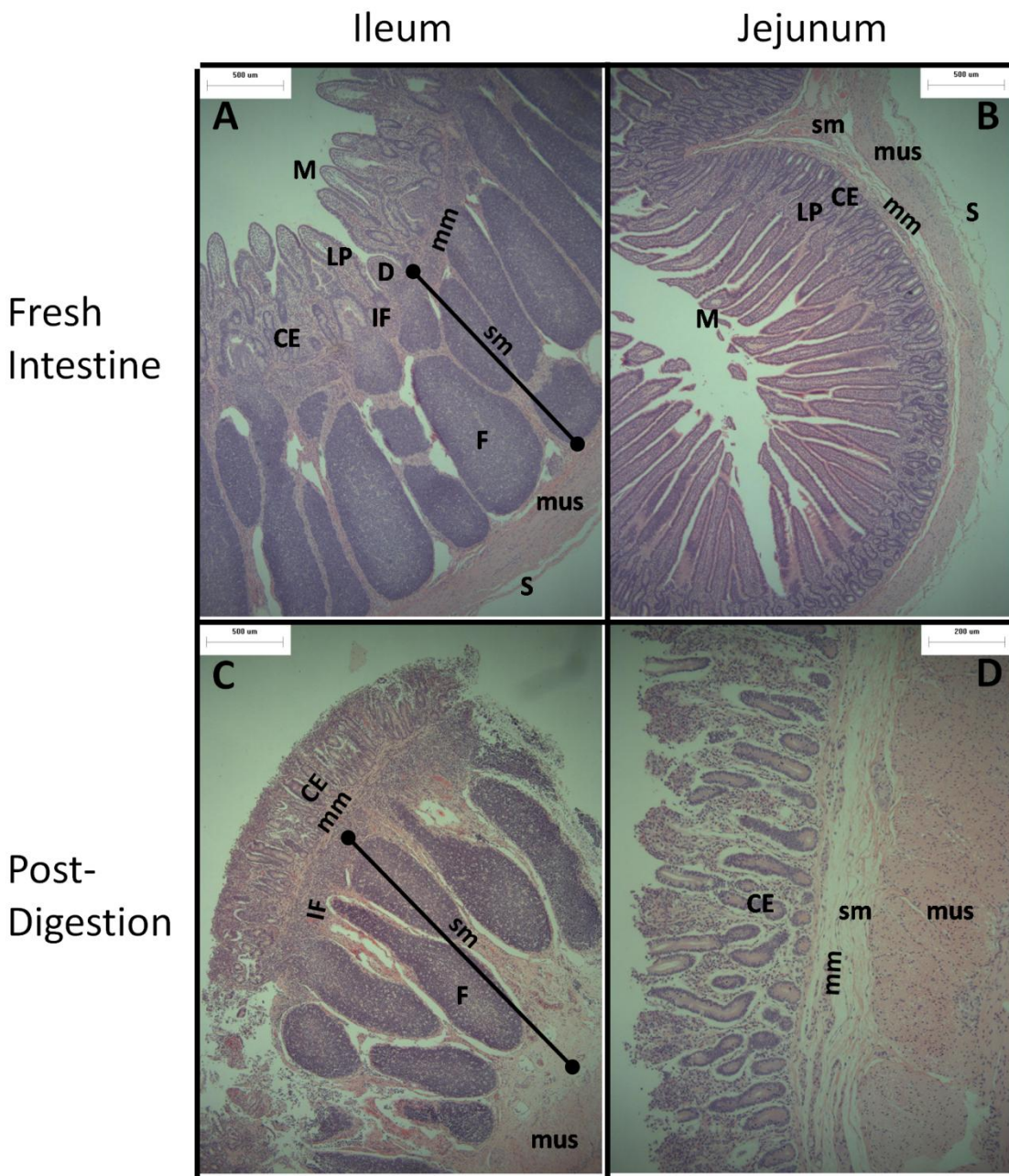


Figure 4.1 Ileal and jejunal tissue before and after enzymatic digestion. Images are magnified 4x. M = mucosa; LP = lamina propria; D = dome; CE = crypt epithelium; IF = inter-follicular region; sm = sub-mucosa; F = follicle; mm = mucosal muscularis; mus = muscularis externa; and S = serosa.

4.3.5 IEL and LPL isolation

The tissue digest protocol used was based on previous procedures with minor modifications [111, 213]. Following the initial 60 minute collagenase digestion, the release of IEL and LPL was completed by first incubating tissue fragments for 120 minutes at 37° C and shaking at 150 RPM in CMF-HBSS, 5% FBS, 1X antibiotic/antimycotic, 30 µg/ml DNase I, and 200U/ml collagenase 4 (Figure 4.2). The resulting supernatant was collected and fresh medium added for a further 60 minute incubation using the same conditions. The supernatant from both digests was pooled, cells were pelleted by centrifugation at 4° C for 8 minutes at 300g, and the cell pellet was re-suspended in PBSA and layered over a 60% Percoll gradient. The gradient was centrifuged at 22° C for 20 minutes at 2000 X g with no brake. Cells at the Percoll-PBSA interphase were collected, washed three times with PBSA, and kept on ice until used for FACS analysis, culture, cytopins, and high speed cell sorting.

4.3.6 PBMC isolation

Blood was collected from the jugular vein using EDTA as an anticoagulant and PBMC were isolated by following the protocol described by Arsenault et al [214]. Blood was collected from calves immediately prior to euthanasia to ensure age-matched samples of PBMC and mucosal leukocytes.

4.3.7 Flow cytometry

Intestinal cells and PBMCs were resuspended at a final concentration of 20×10^6 cells/ml in PBSA containing 0.03% sodium azide (EMD Chemicals). A 50 μ l aliquot of cells was added to each well of a U-bottom 96-well plate (Corning Inc. Life Sciences, Lowell, MA) and a 5 μ l aliquot of primary monoclonal antibody (mAb; Table 3.1) was added at a final concentration between 1-5 μ g/ml. For each mAb the concentration giving maximum labeling intensity without detectable background staining was determined in a titration experiment. Cells were incubated on ice for 20 minutes and then washed 3x by adding 200 μ l PBSA/0.03% sodium azide. Fluorochrome-conjugated goat anti-mouse immunoglobulin (Ig) isotype-specific secondary antibodies (Life Sciences) were then added at a final concentration of 1 μ g/ml and cells were incubated in the dark for 20 minutes on ice. Cells were then washed 3x with 200 μ l PBSA/0.03% sodium azide before fixation with 200 μ l 2% formaldehyde (Sigma-Aldrich). Cells were stored at 4°C in the dark until analyzed with a FACS Calibur (Becton-Dickinson, Franklin Lakes, NJ) using CellQuest acquisition and analysis software (version 3.3). A minimum of 30,000 CD45⁺ events were captured for each sample with data collected in list-mode. Cells were gated to include only CD45⁺ cells and cells that fell within 200-700 forward scatter (FSC) and 50-900 side scatter (SSC). Compensation was FL1 – 0.9% FL2, FL2 – 33% FL1. FL1 uses a 488nm laser with a 530nm filter to detect fluorescein isothiocyanate (FITC) and FL2 uses a 488nm laser with a 585nm filter to detect phycoerythrin (PE). FL4 uses a 635nm laser with a 661nm filter to detect allophycocyanin (APC). Fluorochrome-conjugated secondary antibodies were goat anti-mouse IgG1-FITC conjugated, IgG2b-FITC conjugated, IgM-PE conjugated and IgG2a-APC conjugated (Life Sciences).

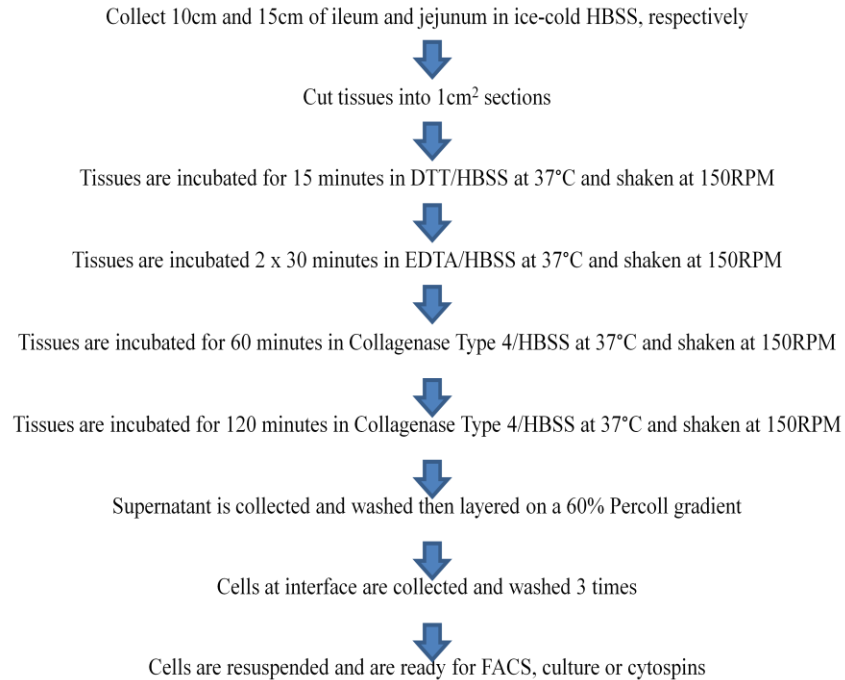


Figure 4.2. Protocol outline for isolating LPL from the ileum and jejunum of calves. Tissues were incubated with DTT, EDTA, and collagenase type 4. Isolated cells were then washed with PBSA, layered on a 60% Percoll gradient and washed 3 more times with PBSA. The cells were then used for FACS, cell culture or cytopins.

4.3.8 Sorting CD11c^{Hi}MHCII⁺ cells

LPL were isolated and cultured overnight in AIM-V media (Life Sciences) containing 5% FBS and 1X antibiotic/antimycotic before collecting both plastic adherent and non-adherent cells. Cells were washed once with PBSA, re-suspended at a final concentration of 108 cells/ml, and incubated with primary monoclonal antibodies specific for bovine CD11c and MHC Class II (Table 4.1) at a final concentration of 4.0 µg/ml for 30 minutes. The cells were washed 3x with PBSA, resuspended at a final concentration of 108 cells/ml and incubated with the appropriate FITC and PE conjugated goat anti-mouse Ig isotype specific antibodies at a final concentration of 4.0 µg/ml for 30 minutes. The cells were washed 3x, resuspended in PBSA and CD11c^{Hi}MHCII⁺ cells were sorted using a FacsVantage (Becton-Dickinson) cell sorter. CD11c^{Hi}MHCII⁺ cells were sorted twice to achieve a purity greater than 99.5%.

Table 4.1: Monoclonal antibodies used for flow cytometry and IHC.

CD ¹	Target Molecule	Target Population	mAb Clone	Supplier
CD1b	CD1b molecule	DC	TH97A	VMRD
CD3	TcR ² complex	pan-T cell	MM1A	VMRD
CD4	CD4 molecule	T _H cells, DC	CACT183A	VMRD
CD8	CD8 α	CTLs ³ , DC	CACT80C	VMRD
CD11b	α chain of CD11b/ CD18	Myeloid	MM10A	VMRD
CD11c	Integrin α X	DC, mono ⁴ ,	BAQ153A	VMRD
CD13	Aminopeptidase N	IDC ⁵ , E ⁶	CC81/MCA2338	AbD Serotec
CD14	LPS ⁷ receptor	Mono, M ϕ ⁸	MM61A	VMRD
CD21	CR2 ⁹	naïve B cell	CC21/MCA1424G	AbD Serotec
CD26	DPP4 ¹⁰	DCs, E	CC69/MCA1652G	AbD Serotec
CD45	PTPRC ¹¹	pan-leukocyte	CACTB51A	VMRD
CD152	CTLA 4 ¹²	DC	IDAC1288	ID Labs
CD172a	SIRPA ¹³	Myeloid	CC149/MCA2041G	AbD Serotec
CD205	LY75 ¹⁴	DC	CCD98/MCA1651F	AbD Serotec
CD335	NCR1 ¹⁵ , NKp46 ¹⁶	NK ¹⁷ cell	MCA2365	AbD Serotec
sIgM	surface IgM	B cell	PIG45A	VMRD
$\gamma\delta$ TcR	TcR1-N24 δ chain	$\gamma\delta$ T cell	GB21A	VMRD
MHC II	MHC Class II molecule	B cell, DCs, activated T cell	H34A	VMRD
IgG1cntl	IgG1 control		MG100	Invitrogen
IgG2a cntl	IgG2a control		MG2a00	Invitrogen
IgG2b cntl	IgG2b control		MG2b00	Invitrogen
IgM cntl	IgM control		MGM00	Invitrogen

1. Cluster of Differentiation; 2. T cell receptor; 3. Cytotoxic T lymphocytes; 4. Monocytes; 5. Interdigitating DC; 6. Epithelial cells; 7. Lipopolysaccharide; 8. Macrophage; 9. Complement Component Receptor 2; 10. Dipeptidyl-peptidase 4; 11. Protein Tyrosine Phosphatase, Receptor Type, C; 12. Cytotoxic T-Lymphocyte Antigen 4; 13. Signal-regulatory Protein Alpha; 14. Lymphocyte Antigen 75; 15. Natural Cytotoxicity Triggering Receptor 1; 16. Natural Killer Cell p46-related Protein; 17. Natural Killer

4.3.9 Cytospins

CD11c^{Hi}MHCII⁺ cells purified by flow cytometric sorting were resuspended in PBSA containing 0.1% EDTA at a final concentration of 10^6 cells/ml. Glass slides (Superfrost Plus, VWR) were pre-coated in the cytospin (Cytospin 4, Fisher Scientific, Waltham, MA) with 25µl FBS by centrifuging slides at 300 RPM for 3 minutes. A 100µl aliquot of cell suspension was then added to the cytospin funnel and centrifuged at 300 RPM for 3 minutes. Slides were air-dried overnight and then stained with Hemacolor staining kit (EMD Chemicals). Slides were again air-dried overnight before mounting coverslips with Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI).

4.3.10 Immunohistochemistry

Five mm² sections of ileum and jejunum were collected immediately after euthanasia and placed with the mucosal surface supported by a 3-4 mm thick section of fresh liver and the serosal surface supported by a strip of acetate film. The tissue block was immersed slowly in liquid N₂ and then stored at -80°C. The IHC procedure was performed as described previously by Griebel et al [215].

4.3.11 Data Analysis

All statistical analyses were performed using GraphPad PRISM software (version 5.04). The assumptions of normal data distribution were verified by examining the equality of the variances of the data (Bartlett's test), the normality of the residuals (Wilks-Shapiro) and constancy of the variance of the residuals (residual plots). Differences among the means of

groups when comparing ileum versus jejunum and individual leukocyte subpopulations were examined using a two-tailed Student's t-Test. P values less than 0.05 were considered significant.

4.4 Results

4.4.1 Isolation of mucosal leukocytes

Histological differences between ileal and jejunal tissues are demonstrated in Figure 4.1. The ileum is characterized by a continuous aggregate of submucosal lymphoid follicles (Figure 4.1A) that occupy almost the complete circumference of the small intestine (data not shown). Therefore, it was not possible to collect ileal tissue devoid of lymphoid follicles which contain primarily sIgM⁺ B cells [216]. In contrast, the jejunal tissue, collected between the discrete PPs, was devoid of organized lymphoid tissue (Figure 4.1B). Ileal tissue also contained organized lymphoid tissue (dome) which extended above the muscularis mucosa (Figure 4.1A). Enzymatic digestion of both ileum (Figure 4.1C) and jejunum (Figure 4.1D) resulted in removal of the absorptive mucosal epithelium, destruction of villi, and extensive exposure of the LP to the level of the crypt epithelium. Lymphoid follicles in the ileal submucosa were also disrupted at the margins of each tissue fragment (Figure 4.1C). Therefore, follicular lymphocytes were present in the cell suspensions recovered following ileal digestion.

It has previously been assumed that IEL and LPL are the only cells isolated following density gradient purification of cells released by enzymatic digestion of intestine [111, 213]. To confirm this we analyzed the expression of cytokeratin which is present in the cytoplasm of mucosal epithelial cells [217], and CD45, a leukocyte common antigen [218]. This analysis revealed that close to half the cells isolated from the ileum were epithelial cells (Figure 4.3A) and a complementary percentage were leukocytes (Figure 4.3B). A similar mix of mucosal

epithelial cells and leukocytes was also isolated from the jejunum (Figure 4.3A & 4.3B). To ensure that flow cytometric analysis of mucosal leukocyte population composition was consistent and accurate the flow cytometric analyses were restricted to CD45⁺ cells.

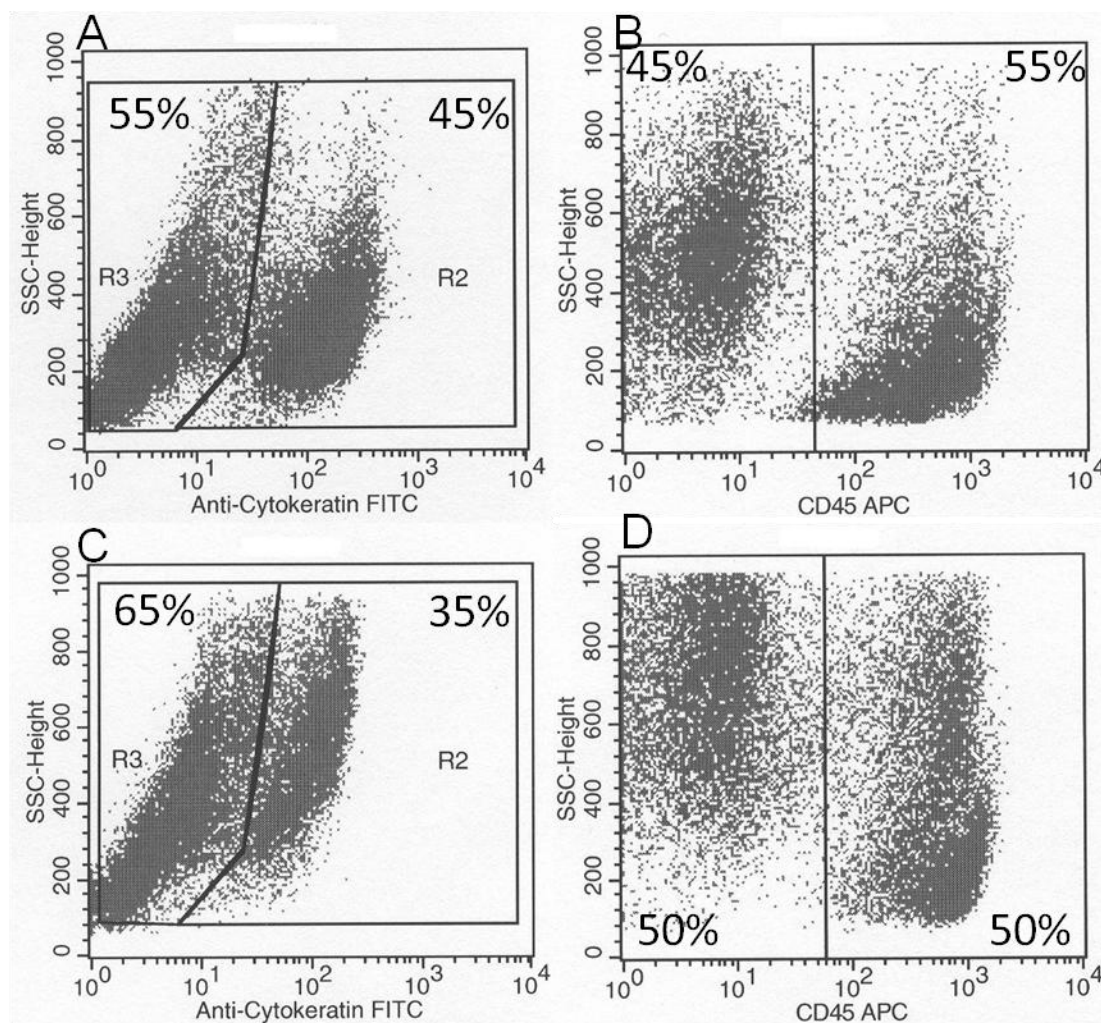


Figure 4.3. Cytokeratin and CD45 expression by cells isolated following EDTA and collagenase digestion of ileal and jejunal tissue fragments. Profiles presented are dot-scatter plots of individual cells with light side scatter (SSC; cell granularity) presented on the Y-axis and either cytoplasmic staining for cytokeratin (anti-cytokeratin-FITC) or surface staining for CD45 (CD45-APC) presented on the X-axis. **(A)** Ileal cells stained for cytokeratin with positive cells gated in R2. **(B)** Ileal cells stained for CD45 with positive population gated to the right. **(C)** Jejunal cells stained for cytokeratin with positive cells gated in R2. **(D)** Jejunal cells stained for CD45 with positive population gated to the right. The percentage negative and positive cells are presented within each panel.

4.4.2 Excluding follicular B cells from the analysis of ileal mucosal leukocytes

Histological examination of digested ileal tissue (Figure 4.1C) indicated that follicular B cells would be present in mucosal leukocyte cell suspensions. Flow cytometric analysis of ileal mucosal leukocytes revealed that 35% to 68% of the cells were sIgM⁺CD21⁺ B cells and IHC staining of ileal tissue confirmed sIgM⁺ B cells were located in the submucosal follicles and dome region (data not shown). The presence of follicular B cells resulted in a relative dilution of all other leukocyte populations and biased comparisons of ileal and jejunal leukocyte subpopulations. To correct for the B cell dilution effect, the frequency of each ileal mucosal leukocyte subpopulation identified with flow cytometry was calculated using the following formula: $[(x-c)/(100-b)] \times 100$ where x = the percentage of positive cells within a specific analysis region labeled with the appropriate lineage specific mAb; c = the percentage of positive cells within the same analysis region labeled with the appropriate isotype control mAb; b = the percentage of sIgM⁺CD21⁺ B cells present in the ileal tissue after subtracting the isotype control value. Data generated with this formula eliminated the effect of contaminating follicular B cells and made it impossible to compare mucosal leukocyte populations within both ileum and jejunum, which did not contain lymphoid follicles. We then calculated the total number of LPL isolated from ileum and jejunum (n = 8). An average of 150×10^6 CD45⁺ cells (Range = 50×10^6 to 340×10^6 cells) was isolated from a 10cm section of ileum, following exclusion of sIgM⁺CD21⁺ B cells. Similarly, an average of 160×10^6 CD45⁺ cells (Range = 70×10^6 to 260×10^6 cells) was isolated from a 10cm section of jejunum. Therefore, similar numbers of mucosal leukocytes were isolated from an equivalent amount of ileal and jejunal tissue.

4.4.3 CD11c^{Hi}MHC Class II⁺ cells define DC

Previous studies in mice and humans defined CD11c^{Hi}MHC Class II⁺ cells as DCs but it is not known if these two markers can be used to identify bovine DCs or myeloid cells[219]. A distinct population of CD11c^{Hi}MHC Class II⁺ cells was apparent within the CD45⁺ leukocytes isolated following digestion of ileum (Figure 4.4A). Furthermore, gating on these CD11c^{Hi} cells revealed that only a small percentage co-expressed CD3, CD21, or CD335. The presence of a small number of CD11c^{Hi}CD3⁺ is consistent with previous reports of these cells in the mesenteric lymph node and PP [186]. Therefore, we concluded that the majority of CD11c^{Hi}MHC Class II⁺ cells belonged to the myeloid lineage. To confirm this conclusion, CD11c^{Hi}MHC Class II⁺ cells from both ileum and jejunum were purified using high speed cell sorting and the phenotype of the cells was analyzed in cytopins by examining 200 cells/slide. Over 95% of the cells from both ileum (Figure 4.4B) and jejunum (Figure 3.4C) were characterized by the presence of abundant cytoplasm, cytoplasmic vacuoles, and many of the cells displayed a variety of cytoplasmic processes or projections. Few cells were seen with a narrow rim of cytoplasm surrounding the nucleus, a phenotype consistent with lymphocytes.

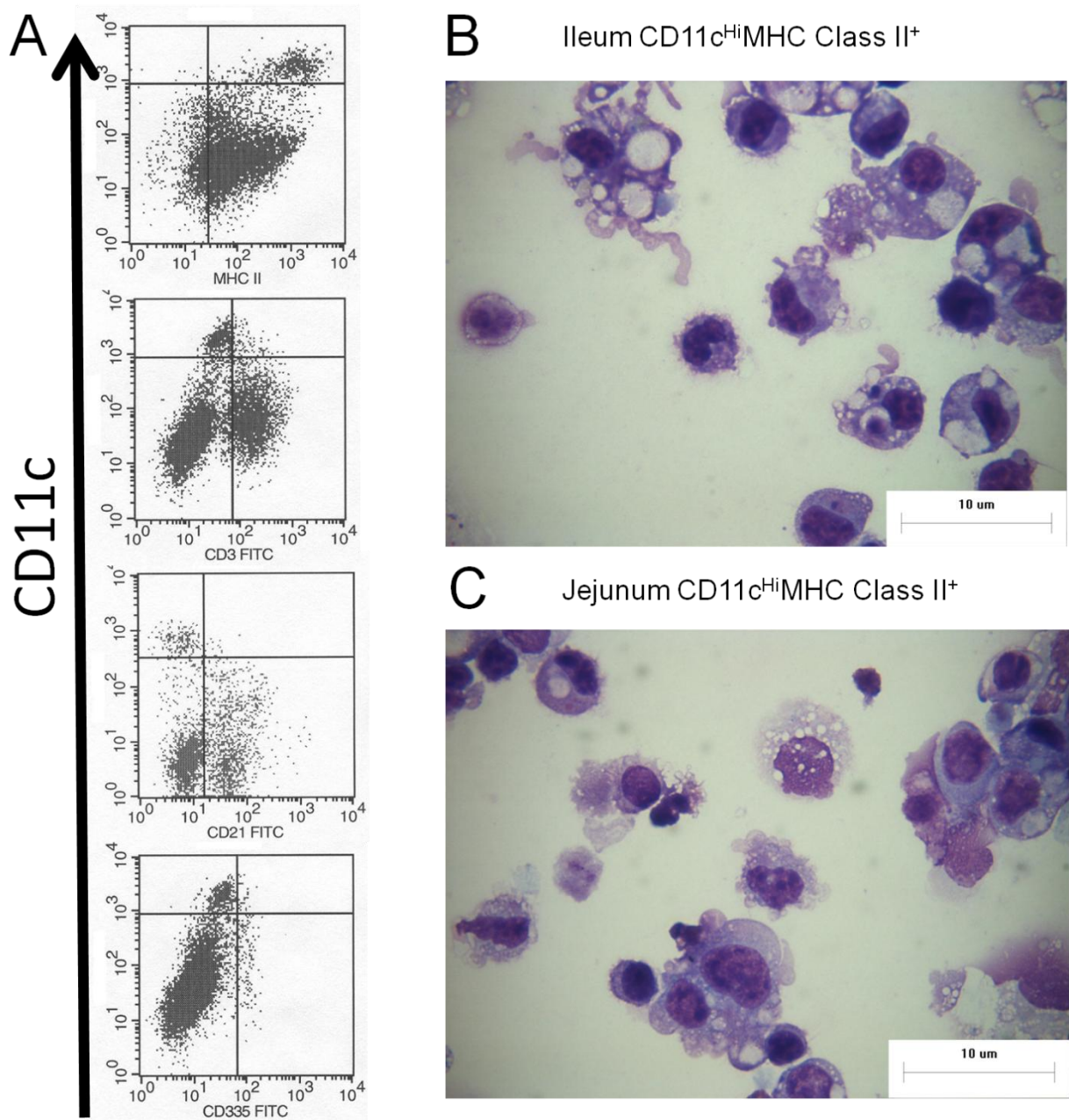


Figure 4.4. CD11c expression on ileal CD45⁺ LPL and the morphology of CD45⁺CD11c^{Hi}MHCII⁺ LPL isolated from ileum and jejunum. **(A)** Dot-scatter plots of CD45⁺ LPL co-stained for CD11c (Y-axis) and either MHC Class II (MHC II) or leukocyte lineage markers for T cells (CD3), B cells (CD21) and NK cells (CD335) (X-axis). **(B)** Giemsa stained cytospin of CD45⁺CD11c^{Hi}MHCII⁺ cells purified by high speed cell sorting following release of

cells from ileal tissue digested with EDTA and collagenase. Image is magnified 100x. (C)
Giemsa stained cytopsin of CD45⁺CD11c^{Hi}MHCII⁺ cells purified by high speed cell sorting
following release of cells from jejunal tissue digested with EDTA and collagenase. Images are
magnified 100x.

4.4.4 Distinct CD11c^{Hi} subpopulations in PBMC and the intestine of newborn calves

Distinct mucosal DC subpopulations have been identified in mice and humans[21]. Therefore, monoclonal antibodies (mAbs) specific for macrophage and DC surface proteins (Table 4.1) were used to analyze the phenotype of CD11c^{Hi} cells circulating in blood and isolated from the small intestine. Flow cytometric analysis confirmed there was a large, distinct population of CD11c^{Hi}MHC Class II⁺ cells present in neonatal blood (Figure 4.5A) and co-staining for CD11c^{Hi} versus either CD3, CD21, or CD335 confirmed this population contained few T cells (Figure 4.6), B cells, or NK cells (data not shown). Therefore, gating on CD11c^{Hi} cells enabled us to analyze myeloid cell populations in both blood and the small intestine. Co-staining for CD11c^{Hi} and putative monocyte/macrophage lineage markers, such as CD14 and CD11b, revealed that virtually all CD11c^{Hi} cells co-expressed these markers in blood but only a subpopulation of intestinal CD11c^{Hi} cells co-expressed CD14 and CD11b. In contrast, putative DC lineage specific markers, such as CD13, CD26, and CD205 were virtually absent on blood CD11c^{Hi} cells but many intestinal CD11c^{Hi} cells co-expressed CD13 and CD205. A quantitative comparison of the differences between blood (n =5) and intestinal tissue (n = 8) was made by calculating the relative abundance of each myeloid subpopulation as a percentage of the total CD11c^{Hi}MHC Class II⁺ population in each tissue (Figure 4.6). This comparison confirmed that monocyte/macrophages (CD11b⁺, CD14⁺, CD172a⁺) were significantly more abundant in blood than intestine and DCs (CD13⁺, CD205⁺) were significantly more abundant in the intestinal mucosa than blood.

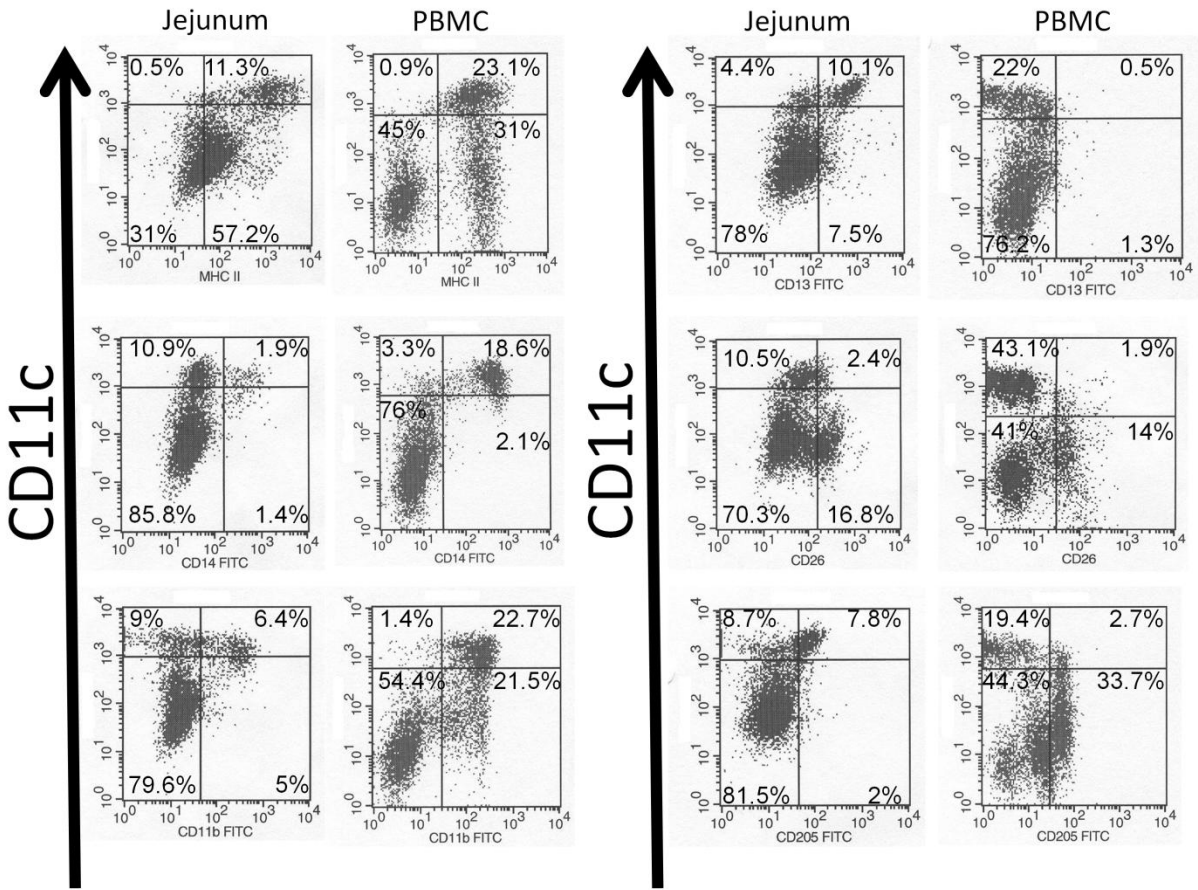


Figure 4.5. Comparison of myeloid cell subpopulations in blood and jejunum. Mononuclear leukocytes were isolated from the buffy coat of blood (PBMC) with a one-step Percoll gradient and CD45⁺ LPL were isolated from jejunum with EDTA and collagenase digestion. Profiles presented are dot-scatter plots of CD11c^{Hi} PBMC and CD45⁺ CD11c^{Hi} LPL (Y-axis) analyzed for co-expression of MHC Class II (MHC II) and a variety of myeloid cell markers including CD14, CD11b, CD13, CD26, and CD205 (X-axis).

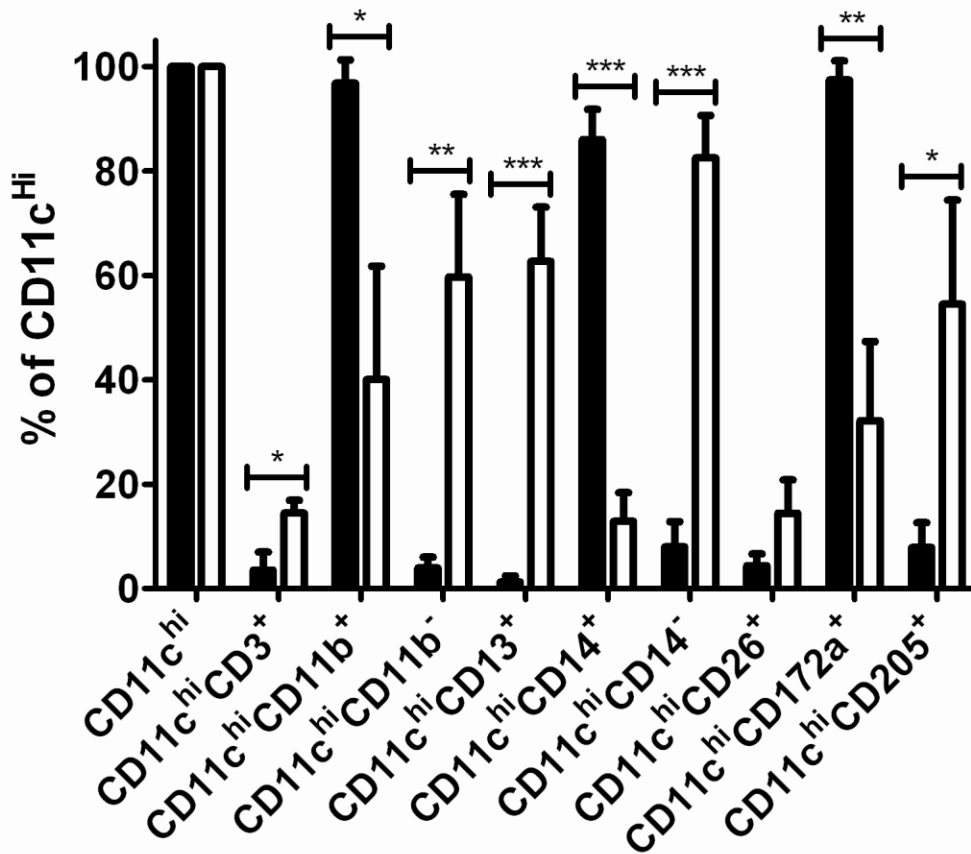


Figure 4.6. Myeloid subpopulation in blood and the small intestine of young calves. Mononuclear cells were isolated from the buffy coat of blood (solid bars) with a one-step Percoll gradient and CD45⁺ LPL were isolated from jejunum with EDTA and collagenase digestion. The total myeloid population in blood (solid bars) was defined as all CD11c^{Hi}MHC II⁺ cells (100%) and each myeloid subpopulation was calculated as a percentage of this population. The total myeloid population in jejunal LPL (open bars) was defined as all CD45⁺CD11c^{Hi}MHC II⁺ cells (100%) and each myeloid subpopulation was calculated as a percentage of this population. Data presented are the mean and 1 SD of values from blood (n = 5) and jejunal LPL (n = 8). Differences between myeloid populations in blood and jejunal LPL were examined using a two-tailed Student's t-Test and significant differences are indicated: * p < 0.05; ** p < 0.01; ***p < 0.001

4.4.5 Regional differences in mucosal myeloid and lymphoid populations

Ileal and jejunal PPs in newborn calves display distinct structural and functional characteristics [201]. Therefore, we investigated whether regional differences in organized MALT were reflected in the leukocyte populations within the mucosa and LP. No significant differences were observed when comparing total T cells ($CD3^{+}$) or individual T cell subpopulations but $CD45^{+}CD335^{+}$ NK cells were significantly more abundant in ileum than jejunum (Figure 4.7A). There was a similar abundance of total $CD11c^{Hi}MHC\ Class\ II^{+}$ cells when comparing ileum and jejunum (Figure 4.7A). Analysis of individual myeloid subpopulations, as a percentage of the total mucosal leukocyte population, revealed that the only significant difference between ileum and jejunum was a greater abundance of $CD45^{+}CD11c^{Hi}CD14^{+}$ cells in the ileum (Figure 4.7B). Further analysis of mucosal myeloid subpopulations, expressed as a percentage of the total $CD45^{+}CD11c^{Hi}MHC\ Class\ II^{+}$ population, confirmed that only the $CD11c^{Hi}CD14^{+}$ population was significantly more abundant in the ileum (Figure 4.7C).

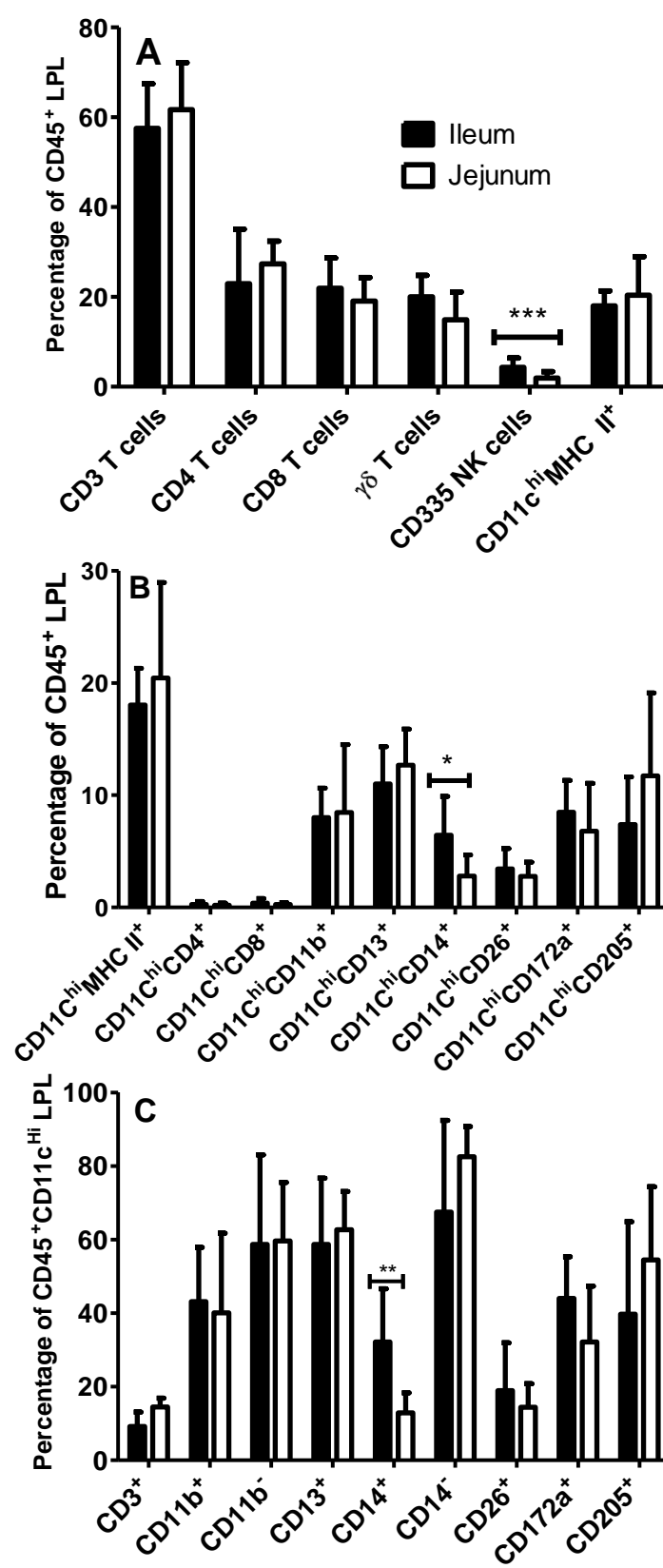


Figure 4.7. Phenotype of mucosal leukocyte subpopulations isolated from the ileum and jejunum. (A) Frequency of major lymphoid and myeloid subpopulations within the total CD45⁺ population. (B) Frequency of myeloid subpopulations within the total CD45⁺ population. (C) Relative frequency of myeloid subpopulation expressed as a percentage of the total myeloid (CD45⁺CD11c^{Hi}) population. Differences between ileal and jejunal leukocyte subpopulations were examined using a two-tailed Student's t-Test and significant differences are indicated are indicated: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

IHC was then used to confirm that the myeloid cells isolated by tissue digestion were present within either the intraepithelial or LP compartments of the mucosa (Table 4.2). IHC revealed differences in myeloid cell distribution within the tissue which were not apparent when analyzing cell suspensions. For example, while CD11c⁺ cells were abundant in the LP of both ileum and jejunum they were only present within the jejunal mucosa (Figure 4.8; Top Panel). In contrast, CD11b⁺ cells were also abundant in the LP of both ileum and jejunum but they were only present within the ileal mucosa (Figure 4.8; 2nd panel from top). IHC confirmed CD14⁺ cells were rare in the LP of the jejunum (Figure 4.8; 3rd panel from top) and revealed that CD26 was also expressed not only by cells within the LP but also on the surface of mucosal epithelium (Figure 4.8; 4th panel from top). Finally, staining for CD172a confirmed that cells expressing this marker were present in the LP of ileum and jejunum but absent from the epithelial layer. Cells expressing putative macrophage markers (CD11b, CD14, and CD172a) were present in the submucosa of both ileum and jejunum but cells expressing putative DC markers (CD11c, CD13, and CD26) were absent or rare in the submucosa of the jejunum.

Table 4.2: Distribution of lymphoid and myeloid cells in neonatal intestinal tissue.

Cell Type	ILEUM						JEJUNUM		
	IEL ¹	LP ²	DE ³	D ⁴	F ⁵	IF ⁶	IEL ¹	LP ²	SM ⁷
CD4 ⁺	-	+	R ⁸	+	R	+	-	+	-
CD8 ⁺	+	R	R	R	R	+	+	R	-
$\gamma\delta$ TcR ⁺	+	R	-	-	-	-	+	R	-
CD11b ⁺	+	+	+	+	+	+	-	+	+
CD11c ⁺	-	+	+	+	R	+	+	+	-
CD13 ⁺	+	+	+	+	+	+	+	+	-
CD14 ⁺	-	+	-	+	+	+	-	+	+
CD26 ⁺	-	+	+	+	+	+	-	+	R
CD172a	-	+	-	+	+	-	-	+	+

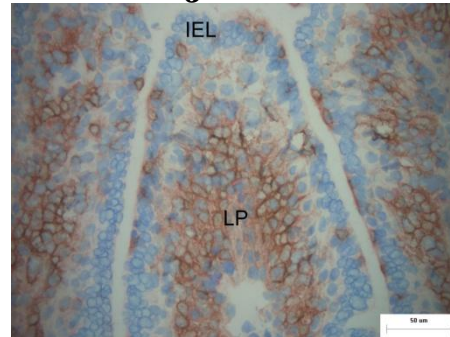
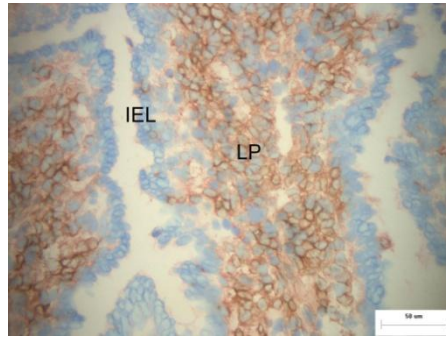
1. Intraepithelial leukocyte; 2. Lamina propria; 3. Dome epithelium; 4. Dome region of PP

follicle; 5. Lymphoid follicle in PP; 6. Interfollicular region in PP. 7. Submucosa; 8. Rare

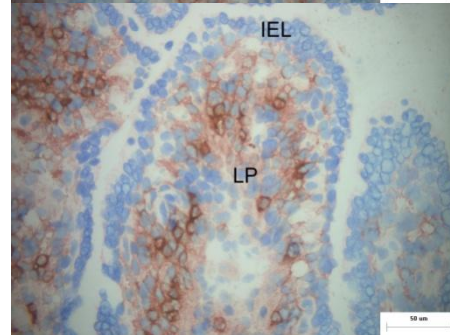
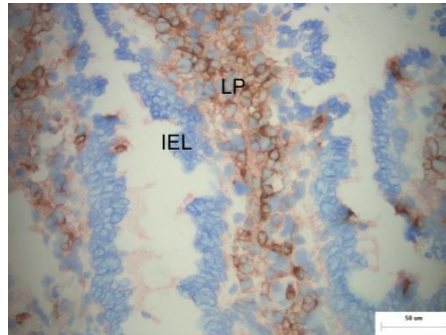
Ileum

Jejunum

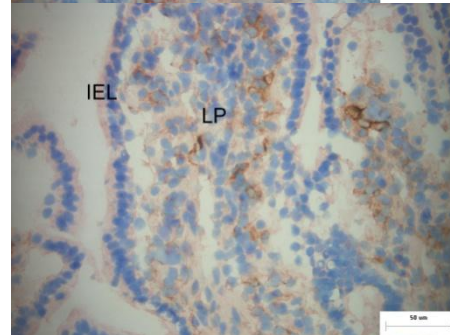
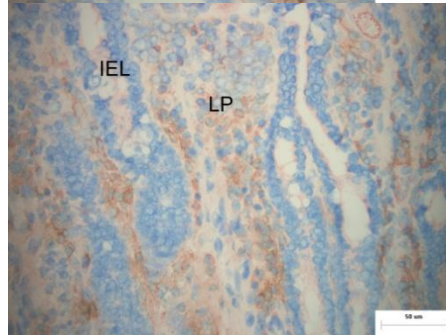
CD11c



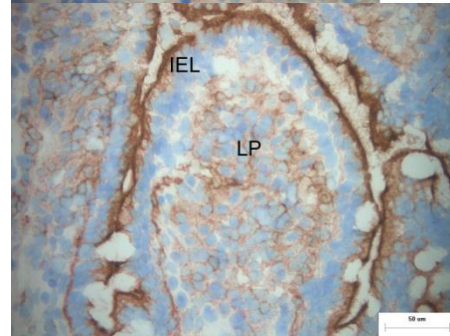
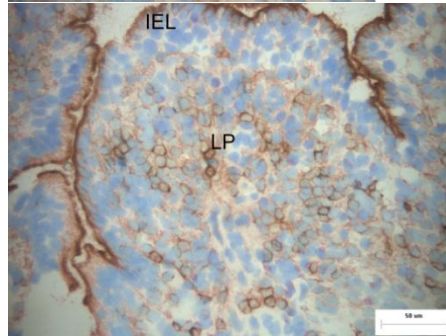
CD11b



CD14



CD26



CD172a

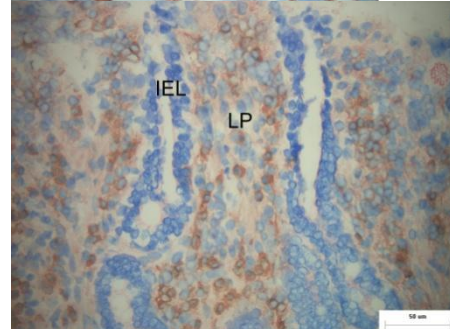
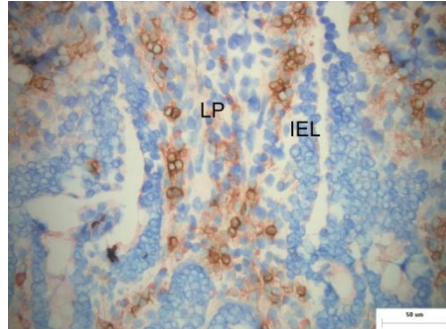


Figure 4.8. Distribution of cells expressing CD11c, CD11b, CD14, CD26, and CD172 in the mucosa and LP of ileum and jejunum collected from young calves. Tissue cryosections were stained with monoclonal antibodies specific for each leukocyte antigen and staining was visualized with 3,3'-diaminobenzidine substrate (brown cells). Irrelevant isotype-matched monoclonal antibodies were used to control for non-specific staining and no visible staining was observed. Images are magnified 40X and the mucosal epithelium (IEL) and lamina propria (LP) are indicated.

4.4.6 T cell distribution in ileum and jejunum

CD8 and $\gamma\delta$ TcR T cells are abundant but CD4 T cells are absent within the IEL compartment of the small intestine of young calves [220]. Thus, monitoring T cell subpopulations within intestinal tissue digests provides an effective way to determine whether cells are isolated from the IEL or LPL compartments. IHC analysis of ileum and jejunum confirmed CD4 T cells were abundant in the LP but absent within the mucosal epithelium (Figure 4.9; Top Panel). Furthermore, while CD8 and $\gamma\delta$ TcR T cells were relatively rare in the LP, these T cell subpopulations were also abundant within the mucosa of both the ileum and jejunum (Figure 4.9: middle and bottom panel; Table 4.2). Therefore, the relatively equal abundance of CD4, CD8 and $\gamma\delta$ TcR T cells in the mucosal leukocyte populations isolated by tissue digest (Figure 4.7A) confirmed that the isolated cells included both IEL and LPL.

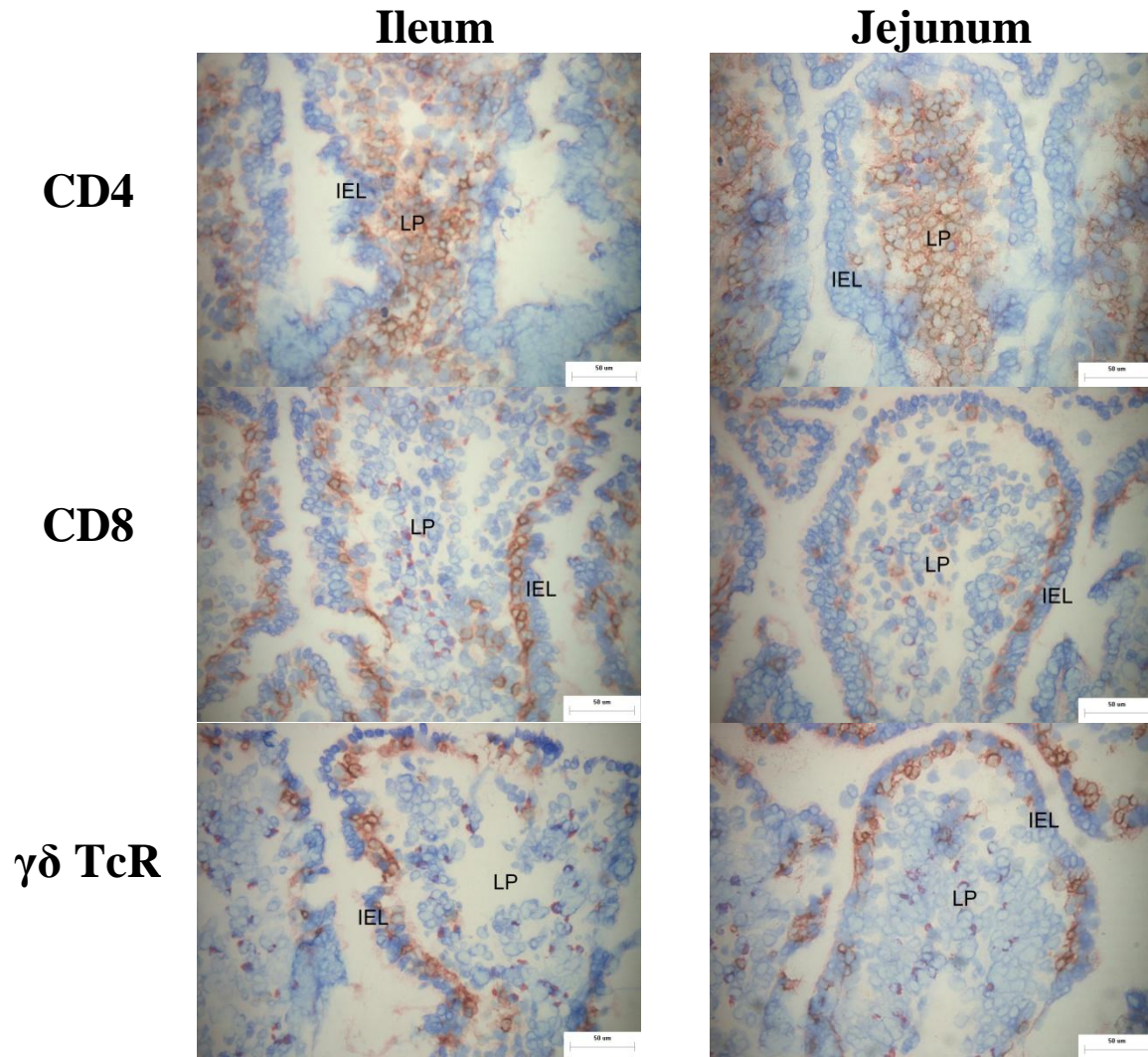


Figure 4.9. The distribution of mucosal T cell subpopulations expressing CD4, CD8, and the $\gamma\delta$ TcR in the ileum and jejunum of young calves. Tissue cryosections were stained with monoclonal antibodies specific for each leukocyte antigen and staining was visualized with 3,3'-diaminobenzidine substrate (brown cells). Irrelevant isotype-matched monoclonal antibodies were used to control for non-specific staining and no visible staining was observed. Images are magnified 40X and the mucosal epithelium (IEL) and lamina propria (LP) are indicated.

4.5 Discussion

Phenotypically distinct DC subpopulations have been identified throughout the GIT of mice and humans with distinct DC subpopulations located in the oral cavity, gastric mucosa, and small intestine [138-142, 144-147, 154]. The development and distribution of these distinct mucosal DC subpopulations has not, however, been analyzed in the newborn. These studies are critical because the mucosal immune system provides the first defense barrier for over 90% of the pathogens and commensal microflora that colonize the GIT of the newborn within hours of birth. Furthermore, it has been difficult to compare DC phenotype among species due to the frequent use of species-specific reagents. Some DC molecules are conserved across species and both CD11b and CD11c are found on murine, human, and bovine DCs [138-140, 187, 201]. In the present investigation we confirmed that cells expressing a high level of CD11c and MHC Class II were myeloid cells (Figure 4.4). It was then possible to analyze the diversity of CD11c^{Hi} myeloid cell populations in the small intestine and blood of newborn calves to determine if distinct mucosal DC populations are present early in life with regional differences in DC distribution within the small intestine.

Marked phenotypic differences were observed when comparing mucosal and blood myeloid cell populations. The distinct phenotype of mucosal myeloid cells was highlighted by the absence CD11c^{Hi}CD13⁺ cells in blood (Figure 4.5) and significant differences in the abundance of other myeloid subpopulations (Figure 4.6). Previous studies used differential expression of CD14 on CD11c⁺ cells to distinguish bovine monocyte and DC populations in blood and confirmed that DCs were relatively rare in blood [210, 221]. Our analysis supports this conclusion with the majority of blood CD11c^{Hi} cells co-expressing CD14, CD172a, and CD11b. In contrast, these monocyte/macrophage markers were expressed on relatively minor

subpopulations of mucosal myeloid cells and the majority of CD11c^{Hi} cells expressed CD13 and CD205 (Figure 4.6). These observations suggest a significant DC enrichment in the intestine of newborn calves when compared to blood. These DC subpopulations may reflect either selective recruitment from blood or differentiation under the influence of the local microenvironment. The relatively low frequency of macrophages (CD11c^{Hi} and either CD14⁺, CD172a⁺, or CD11b⁺) in the intestine may reflect limited monocyte recruitment from blood if we assume expression of these molecules is maintained in the tissue. In contrast, the absence of CD11c^{Hi}CD13⁺ cells in blood supports the conclusion that local differentiation is at least one factor contributing to the development of distinct mucosal DC populations.

Previous studies of the intestinal mucosal immune system in newborn calves revealed a rapid accumulation of T cells in the LP and mucosa of the ileum during the first three weeks of life [118]. It was not determined whether this T cell development was consistent throughout the small intestine. Our investigation confirmed that all T cell subpopulations are abundant in the mucosal and LP by 3 to 5 weeks of age (Figure 4.9) and are distributed with a similar frequency in both ileum and jejunum (Figure 4.7A). Analysis of NK cells revealed, however, that significant regional differences can exist in the development of mucosal lymphoid populations. In contrast, analysis of CD11c^{Hi}MHCII⁺ cells suggested that the distribution of myeloid cells was similar throughout both ileum and jejunum. Immature DCs may be CD11c^{Hi}MHCII⁻ [222] and this population was also apparent in the cell suspensions isolated from the small intestine (Figure 4.5A). A comparison of CD11c^{Hi}MHCII⁻ cell frequency in ileum and jejunum did not, however, reveal a significant difference (data not shown). Therefore, we concluded the overall frequency of CD11c^{Hi} cells was similar throughout the ileum and jejunum. In contrast, when IHC was used to examine the distribution of CD11c⁺ cells within intestinal tissues then regional differences in

DC distribution became apparent. Localization of CD11c⁺ cells within the jejunal epithelium was a striking regional difference and this was complemented by a restricted distribution of CD11b⁺ cells within the ileal mucosa (Figure 4.8). Selective isolation of IEL will be required to confirm that CD11c⁺ and CD11b⁺ cells represent DCs and macrophages, respectively. Furthermore, if CD11c⁺ and CD11b⁺ intraepithelial cells differ in their capacity to sample luminal contents and recognize pathogen-associated molecular pattern molecules then this differential distribution may have significant implications for neonatal immune surveillance and mucosal responses to pathogens and commensal microflora.

Further analysis of the frequency of myeloid subpopulations in the ileum and jejunum revealed only minor differences with the exception of CD11c^{Hi}CD14⁺ cells (Figure 4.7). Analysis of CD11c^{Hi}CD14⁺ frequency, either as a percentage of all CD45⁺ leukocytes (Figure 4.7B) or as a subpopulation of CD45⁺CD11c^{Hi} myeloid cells, confirmed that this macrophage population was more abundant within the ileum. IHC confirmed that CD14⁺ cells were present within the LP of both ileum and jejunum (Figure 4.8) but the presence of PPs within the ileum must also be considered as a possible source of macrophages. CD14⁺ cells were observed within the dome, interfollicular region, and lymphoid follicles of the ileal PP (Table 4.2) and the presence of B cells in ileal cell suspensions indicates that PPs could be a source of CD14⁺ macrophages. Therefore, an apparent difference in the regional distribution of mucosal CD11c^{Hi}CD14⁺ cells may simply reflect regional differences in the distribution of MALT. We would then conclude that mucosal DCs and macrophages are distributed with a similar frequency throughout the small intestine of the newborn but there are marked regional differences in intraepithelial myeloid cells.

A variety of digestion techniques have been developed to selectively isolate LPL and IEL. Wyatt et al. (1996) used a technique whereby the ileum was divided into 10cm segments and then inverted to expose the mucosal surface. They reported that IEL and LPL were isolated as separate populations but the presence of $CD4^+$ T cells and $sIgM^+$ B cells in their IEL population indicates that LPL were also sampled (Figure 4.9). IHC has been used to investigate the phenotype of cells in the small intestine, but this does not allow for reliable quantification of cell types [220, 223] and limits phenotype characterization to co-expression of one or two molecules. We used a tissue digestion technique that released both IEL and LP leukocytes (Figure 4.2) and with this technique it was critical to exclude epithelial cells from the flow cytometric analysis (Figure 4.3). The PPs in the ileum also presented a unique challenge since contaminating B cells (data not shown) can significantly altered the relative frequency of other leukocyte subpopulations in the intestinal cell suspension. Exclusion of epithelial cells and B cells from our analysis was critical to ensure a consistent comparison of ileal and jejunal mucosal leukocyte populations. Previous flow cytometric analyses of ileal leukocytes in mature cattle did not exclude epithelial cells and did not confirm that all cells within the analysis gate were $CD45^+$ leukocytes [111, 118]. Setting these minimal criteria for phenotypic studies of mucosal leukocytes is important to ensure consistent quantification of specific mucosal leukocyte populations. This will be critical when analyzing mucosal responses to infectious agents, commensal microflora or age-related changes in the mucosal immune system.

In conclusion, combined flow cytometric and IHC analysis provided substantial evidence that the intestine of the newborn calf is populated by diverse macrophage and DC subpopulations. DCs were abundant in the newborn small intestine and IHC revealed distinct regional differences in the distribution of these cells, especially within the mucosal epithelium.

Further studies will be required to determine if these regional differences in myeloid cell distribution significantly impact mucosal responses to either commensal microflora or enteric pathogens.

4.6 Conclusions

Understanding mucosal immune system development is critical for understanding neonatal disease and developing mucosal vaccines. We developed an effective method to isolate and analyze mucosal leukocyte subpopulations from the intestine of the newborn calf. The use of CD11c to identify mucosal myeloid cells was validated and subsequent analyses confirmed the neonatal intestine was populated by a unique population of CD11c^{Hi}CD13⁺ DCs. Furthermore, there was a predominance of mucosal DCs versus macrophages within the small intestine. A comparison of ileal and jejunal mucosal leukocytes confirmed there were significant differences in the regional distribution of mucosal leukocytes. These regional differences were apparent in the distribution of NK cells and the intraepithelial distribution of DCs (CD11c⁺) and macrophages (CD11b⁺). This study provides quantitative baseline values for mucosal leukocyte populations in the neonate which will be important for future studies investigating the effects of pathogens or commensal microflora.

CHAPTER 5

AGE-RELATED CHANGES IN THE DISTRIBUTION AND FREQUENCY OF MYELOID AND T CELL POPULATIONS IN THE SMALL INTESTINE OF CALVES

Fries P, Popowych Y, Guan L, Griebel P. *Age-Related Changes in the Distribution and Frequency of Myeloid and T Cell Populations in the Small Intestine of Calves*. Resubmitted to Cellular Immunology (July 22, 2011).

5.1 Abstract

Mucosal dendritic cells (DCs) play a key role in discriminating between dietary antigens, commensal microflora, and pathogens but little is known regarding age-related changes in mucosal DC populations. We analyzed lymphoid and myeloid populations within the epithelium and lamina propria (LP) of the ileum and jejunum of weaned calves (6 months old) and compared their frequency and distribution with newborn calves (3-5 weeks old). CD4, CD8, and $\gamma\delta$ TcR T cells and CD11c^{Hi}MHC Class II⁺ myeloid cell frequency were significantly different when comparing ileum and jejunum of weaned calves. In particular, the number of CD8 and $\gamma\delta$ TcR T cells and CD11c^{Hi}CD14⁺ macrophages was significantly greater in the ileum but CD11c⁺ and CD11b⁺ myeloid cell distribution was similar throughout the mucosal epithelium of the small intestine. Furthermore, significant age-related changes were apparent when comparing the frequency and abundance of mucosal leukocyte subpopulations in newborn and weaned calves. Total mucosal leukocytes (CD45⁺) increased significantly with age in both ileum and jejunum and much of this increase was attributed to mucosal T cells (CD3⁺). In particular, CD4 T cells and NK cells increased significantly in the jejunum and CD8, and $\gamma\delta$ TcR T cells

increased significantly with age throughout the small intestine. In contrast, CD11c^{Hi}MHC Class II⁺ myeloid cells remained numerically unchanged with age but DCs (CD13⁺, CD26⁺, CD205⁺) were enriched and macrophages (CD14⁺,CD172a⁺) were depleted in older animals. Therefore, regional differences between ileal and jejunal mucosal leukocytes changed with age and there was also a marked age-dependent change in the composition of mucosal myeloid cells. These observations have significant implications for host responses to both pathogens and commensal microflora.

5.2 Introduction

The mucosal immune system provides the primary defence against infection by over 90% of pathogens and also provides a critical barrier to the large community of commensal microflora within the gastrointestinal tract (GIT). Many developmental changes occur in the mucosal immune system following birth and evidence from a variety of species indicates that exposure to commensal microflora contributes to these changes and have a profound effect on the development of the entire body. The major organs of gnotobiotic mice do not develop at the same rate as wild type mice [224]. Immunologically, Peyer's patches (PP) are void of germinal centres and as a result, IgA-secreting plasma cells do not develop [89]. Intestinal villi are decreased in height and develop less vascular networking, resulting in decreased intestinal mass and surface area [85]. Furthermore, commensal microflora activate toll-like receptors which provide a range of host benefits [225, 226]. Similar effects on the development of mucosal epithelium and the mucosal immune system have also been observed in gnotobiotic pigs [227].

Dendritic cells (DCs) are a key link between innate and acquired immunity through their role in antigen presentation and regulation of T cell activation. DCs are present at all mucosal

surfaces throughout the body and each mucosal region contains different DC subpopulations. Mucosal DCs interact directly with pathogens and there is increasing evidence that DCs are continually sampling microbes at mucosal surfaces [228]. Furthermore, with over 10^{14} microbial cells residing at mucosal surfaces in the gastrointestinal tract there is increasing evidence that DCs are able to discriminate between pathogenic and non-pathogenic microbes [229]. This is a key component in preventing chronic inflammation but defects in DC function have been linked to allergies and diseases such as ulcerative colitis and Crohn's Disease [145, 170, 230]. Therefore, DC development and distribution at mucosal surfaces in the gastrointestinal tract is a critical component in the interaction between the mucosal immune system, commensal microflora, and pathogens. The phenotype of DCs at individual mucosal sites has been characterized in mice and humans, but little is known about DC phenotype and distribution at mucosal sites in other mammalian species and little is known regarding age-related changes in mucosal DC distribution.

We have previously shown that the small intestine of newborn calves is populated by diverse DC subpopulations with significant regional differences in the distribution of these mucosal DC subpopulations[20]. We also determined that the ileum and jejunum of newborn calves was populated by a distinct population of $CD11c^{Hi}CD13^{+}$ DCs that are absent from blood. However, little is known regarding changes in the phenotype and distribution of intestinal DCs with increasing age and persistent exposure to commensal microflora. Therefore, we analyzed the phenotype and distribution of mucosal lymphoid and myeloid subpopulations in the ileum and jejunum of weaned calves and compared these results with data generated from newborn calves.

These studies confirm that significant regional differences exist within the mucosal immune system of the small intestine but these regional differences change with age. With increasing age, DC subpopulations are enriched within the CD11c⁺MHCII⁺ myeloid population despite a relatively constant number of myeloid cells. A greater understanding of age-related changes in mucosal immune surveillance will, therefore, require the isolation and functional characterization of individual DC subpopulations to determine if functional changes occur despite the relatively constant abundance of these cells.

5.3 Material & Methods

5.3.1 Animals

Castrated male Holstein calves, fed a ration of grain and hay, were purchased from a commercial dairy when they were an average age of 6 months (range = 5.5 to 6.5 months). These calves are referred to as weaned since no milk was included in the diet after 3 months of age. Calves were humanely euthanized with an intravenous injection of Euthanyl[®] (240mg/ml; Bimeda-MTC Animal Health Inc., Cambridge, ON) and tissues were collected within 10 minutes of death. Experiments were conducted according to the Guide to the Care and Use of Experimental Animals, provided by the Canadian Council on Animal Care and all experimental protocols were approved by University of Saskatchewan Animal Care Committee.

5.3.2 Tissue collection

A 10 cm segment of ileal tissue was collected immediately proximal to the ileo-cecal fold and a 15 cm segment of jejunal tissue was collected 3-4 m anterior to the end of the continuous ileum PP and in a region between two discrete PPs. Tissues were placed into ice-cold calcium

and magnesium free Hanks balanced saline solution (CMF-HBSS) supplemented with 5% fetal bovine serum (FBS; SeraCare, Milford, MA) and 1X antibiotic/antimycotic (100U/ml penicillin, 100µg/ml streptomycin and 0.25ug/ml amphotericin B; Life Technologies, Carlsbad, CA) and transported to the lab on ice.

5.3.3 Tissue digestion

Tissue digestion was performed as previously described by Fries et al (In Press). Briefly, intestinal segments were opened, ingesta removed, and the mucosal surface washed with CMF-HBSS. Each intestinal section was cut into 1cm² pieces and transferred to a glass beaker supplemented with 5% FBS and antibiotic/antimycotic.

Dithiothreitol (DTT, 10mM, Sigma-Aldrich, St. Louis, MO) and ethylenediaminetetraacetic acid (EDTA) washes were used to remove mucosal epithelium prior to tissue digestion with collagenase. Following the initial 60 minute collagenase digestion, the release of IEL and LPL was completed by first incubating tissue fragments for 120 minutes at 37° C shaking at 150 RPM in CMF-HBSS, 5% FBS, 1X antibiotic/antimycotic, 30 µg/ml DNase I, and 200U/ml collagenase 4. The resulting supernatant was collected and fresh medium added for a further 60 minute incubation using the same conditions. The supernatant from both digests was pooled, cells were pelleted by centrifugation at 4° C for 8 minutes at 300g, the cell pellet was re-suspended in Ca⁺⁺Mg⁺⁺-free phosphate buffered saline (PBS) and layered over a 60% Percoll gradient. The gradient was centrifuged at 22° C for 20 minutes at 2000g with no brake. Cells at the Percoll-PBS interphase were collected, washed three times with PBS, and kept on ice until used for flow cytometric analysis.

5.3.4 Flow cytometry

Intestinal cells were re-suspended at a final concentration of 20×10^6 cells/ml in PBSA containing 0.03% sodium azide (EMD Chemicals). A 50 μ l aliquot of cells was added to each well of a U-bottom 96-well plate (Corning Inc. Life Sciences, Lowell, MA) and a 50 μ l aliquot of primary monoclonal antibodies (mAb; Table 5.1) was added at a final concentration between 1-5 μ g/ml. The optimal mAb concentration giving maximum labeling intensity without detectable background staining was first determined in titration experiments. Cells were incubated on ice for 20 minutes and then washed 3 times by adding 200 μ l PBSA/0.03% sodium azide. Fluorochrome-conjugated goat anti-mouse Ig isotype-specific secondary antibodies (Life Sciences) were then added at a final concentration of 1 μ g/ml and cells were incubated in the dark for 20 minutes on ice. Cells were washed 3 times with 200 μ l PBSA/0.03% sodium azide, fixed in 200 μ l 2% formaldehyde (Sigma-Aldrich), and stored at 4°C in the dark until analyzed with a FACS Calibur (Becton-Dickinson, Franklin Lakes, NJ) using CellQuest acquisition and analysis software (version 3.3). A minimum of 30,000 CD45⁺ events were captured for each sample and data was collected in list-mode. Cells were gated to include only CD45⁺ cells that fell within 200-700 forward light scatter (FSC) and 50-900 side light scatter (SSC) and compensation was FL1 – 0.9% FL2, FL2 – 33% FL1. FL1 uses a 488nm laser with a 530nm filter to detect fluorescein isothiocyanate (FITC). FL2 uses a 488nm laser with a 585nm filter to detect phycoerythrin (PE). FL4 uses a 635nm laser with a 661nm filter to detect allophycocyanin (APC). Fluorochrome-conjugated secondary antibodies were goat anti-mouse IgG1-FITC, IgG2b-FITC, IgM-PE, and IgG2a-APC (Life Sciences).

Table 5.1: Monoclonal antibodies used for flow cytometry and IHC.

CD ¹	Target Molecule	Target Population	mAb Clone	Supplier
CD1b	CD1b molecule	DC	TH97A	VMRD
CD3	TcR ² complex	pan-T cell	MM1A	VMRD
CD4	CD4 molecule	T _H cells, DC	CACT183A	VMRD
CD8	CD8 α	CTLs ³ , DC	CACT80C	VMRD
CD11b	α chain of CD11b/ CD18	Myeloid	MM10A	VMRD
CD11c	Integrin α X	DC, Mono ⁴ ,	BAQ153A	VMRD
CD13	Aminopeptidase N	IDC ⁵ , E ⁶	CC81/MCA2338	AbD Serotec
CD14	LPS ⁷ receptor	Mono, M ϕ ⁸	MM61A	VMRD
CD21	CR2 ⁹	naïve B cell	CC21/MCA1424G	AbD Serotec
CD26	DPP4 ¹⁰	DCs, E	CC69/MCA1652G	AbD Serotec
CD45	PTPRC ¹¹	pan-leukocyte	CACTB51A	VMRD
CD152	CTLA 4 ¹²	DC	IDAC1288	ID Labs
CD172a	SIRPA ¹³	Myeloid	CC149/MCA2041G	AbD Serotec
CD205	LY75 ¹⁴	DC	CCD98/MCA1651F	AbD Serotec
CD335	NCR1 ¹⁵ , NKp46 ¹⁶	NK ¹⁷ cell	MCA2365	AbD Serotec
sIgM	surface IgM	B cell	PIG45A	VMRD
$\gamma\delta$ TcR	TcR1-N24 δ chain	$\gamma\delta$ T cell	GB21A	VMRD
MHC II	MHC Class II molecule	B cell, DCs, activated T cell	H34A	VMRD
IgG1cntl	IgG1 control		MG100	Invitrogen
IgG2a cntl	IgG2a control		MG2a00	Invitrogen
IgG2b cntl	IgG2b control		MG2b00	Invitrogen
IgM cntl	IgM control		MGM00	Invitrogen

1. Cluster of Differentiation; 2. T cell receptor; 3. Cytotoxic T lymphocytes; 4. Monocytes; 5.

Interdigitating DC; 6. Epithelial cells; 7. Lipopolysaccharide; 8. Macrophage; 9. Complement

Component Receptor 2; 10. Dipeptidyl-peptidase 4; 11. Protein Tyrosine Phosphatase, Receptor

Type, C; 12. Cytotoxic T-Lymphocyte Antigen 4; 13. Signal-regulatory Protein Alpha; 14.

Lymphocyte Antigen 75; 15. Natural Cytotoxicity Triggering Receptor 1; 16. Natural Killer Cell

p46-related Protein; 17. Natural Killer

5.3.5 Immunohistochemistry

Five mm² sections of ileum and jejunum were collected immediately after euthanasia and placed with the mucosal surface supported by a 3-4 mm thick section of fresh liver and the serosal surface supported by a strip of acetate film. The tissue block was immersed slowly in liquid N₂ and then stored at -80°C. The IHC procedure was performed as described previously by Griebel et al [215]. Briefly, indirect labeling of cells was accomplished using primary mAbs and detection with goat anti-mouse Ig isotype-specific biotin-conjugated secondary antibodies. The manufacturers protocol (Vector Labs, Burlingame, CA) was followed for blocking of endogenous peroxidase and non-specific binding of secondary antibodies was minimized by including 2% normal goat serum in all solutions. Non-specific binding of mAbs was monitored with isotype-matched control mAbs (Table 5.1) but no detectable reaction was observed (data not shown). MAb labeling was visualized by using the avidin-biotin complex (ABC) (Vector Labs) and developing with 3,3'-diaminobenzidine substrate.

5.3.6 Data Analysis

All statistical analyses were performed using GraphPad PRISM software (version 5.04). The assumptions of normal data distribution were verified by examining the equality of the variances of the data (Bartlett's test), the normality of the residuals (Wilk-Shapiro) and constancy of the variance of the residuals (residual plots). Differences among the means of groups when comparing ileum versus jejunum and individual leukocyte subpopulations were examined using a two-tailed Student's t-Test. P values less than 0.05 were considered significant.

5.4 Results

5.4.1 Myeloid and Lymphoid Populations in Weaned Calves

Flow cytometric analysis of mucosal leukocyte populations was restricted to CD45⁺ leukocytes since previous analysis confirmed epithelial cells were present in the cell suspensions[20]. Furthermore, ileal, but not jejunal, mucosal leukocyte populations included Peyer's patch (PP) follicular B cells (sIgM⁺,CD21⁺). Therefore, sIgM⁺CD21⁺ cells were subtracted from ileal CD45⁺ leukocytes prior to comparing ileal and jejunal leukocyte populations. Approximately 66% of all cells recovered were CD45⁺ leukocytes (Figure 5.1B). We previously determined that co-expression of CD11c and MHCII⁺ defined myeloid cells within the CD45⁺ mucosal leukocyte population isolated from newborn calves [12]. Analysis of the CD45⁺CD11c^{Hi} population from weaned calves revealed that this population included diverse myeloid populations co-expressing surface molecules associated with macrophages (CD11b, CD14, CD172a) and DCs (CD13, CD26, CD205) (Figure 5.1C). Although CD14⁺ macrophages were relatively rare (1.5 to 2.0%) there were distinct CD11c^{Hi} subpopulations that ranged in frequency from 2.5 to 8.7% (Figure 5.1C). The comparison of major mucosal leukocyte populations isolated from ileum and jejunum revealed significant regional differences in the frequency of both lymphoid and myeloid populations (Figure 5.2). Regional differences in T cell distribution were characterized by a significantly greater CD4⁺ T cell frequency in the jejunum and a significantly greater CD8⁺ and $\gamma\delta$ TcR T cell frequency in the ileum (Figure 5.2A). There was no significant regional difference in NK cell distribution but CD11c^{Hi}MHC Class II⁺ myeloid cells were significantly more frequent in the jejunum (Figure 5.2A). Further analysis of individual CD11c^{Hi} macrophage (CD11b, CD14, CD172a) and DC subpopulations (CD13,

CD26, CD205) did not, however, reveal significant regional differences in the frequency of these populations when comparing ileum and jejunum (Figure 5.2B).

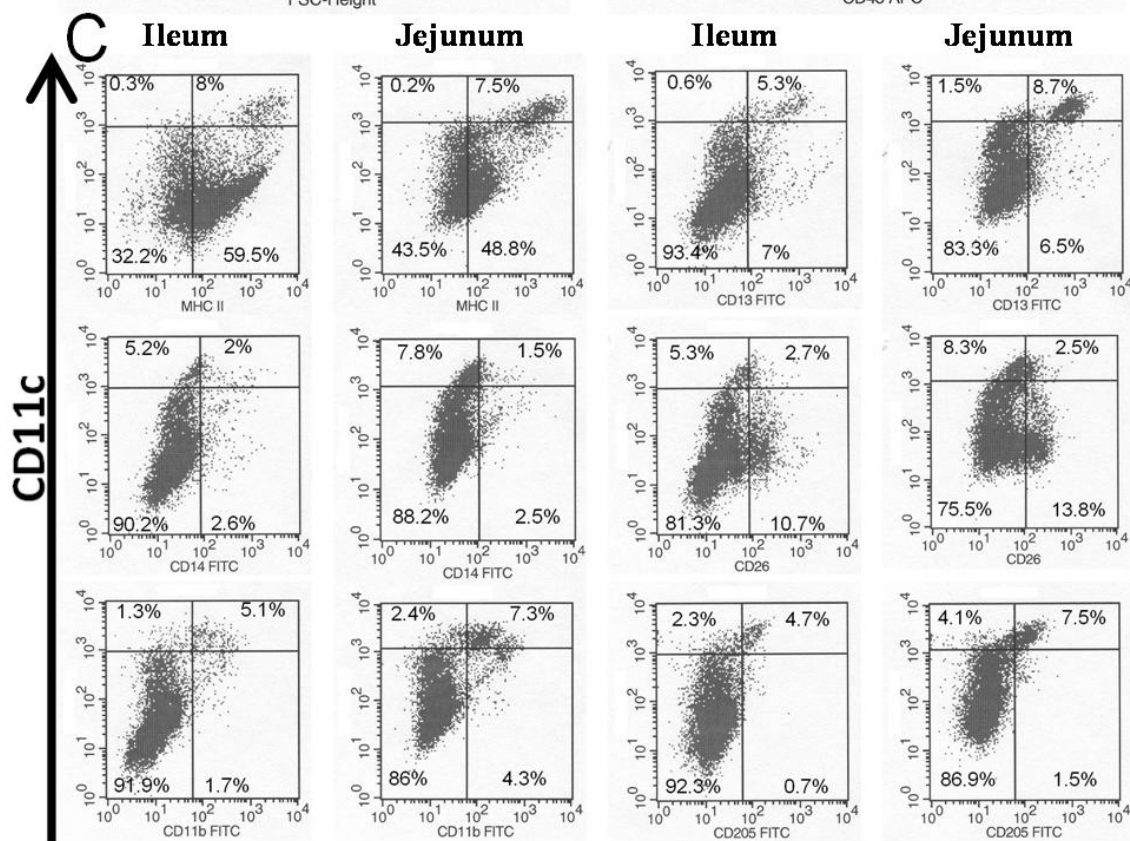
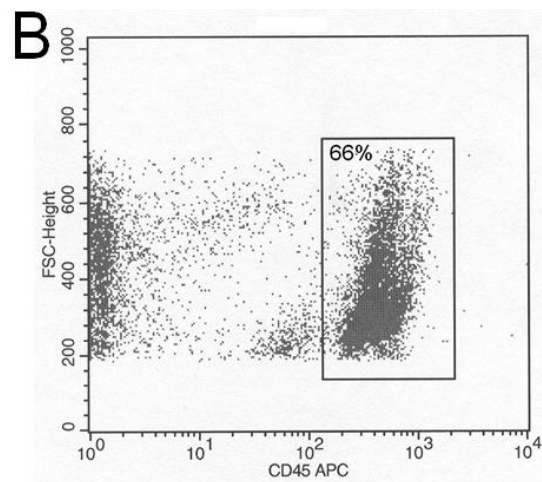
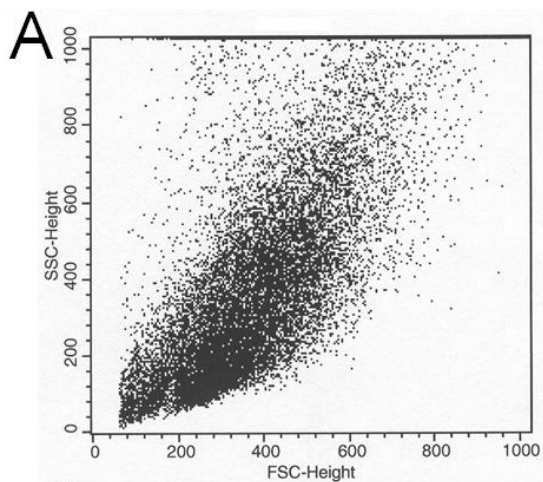


Figure 5.1. Dot-scatter plots comparing mucosal leukocytes isolated from ileum and jejunum by EDTA and collagenase digestion. **(A)** Profile representing the total isolated cell population as defined by side light scatter and forward light scatter. **(B)** Profile showing the region used to select CD45⁺ cells for further analysis. **(C)** Dot-scatter plots are CD45⁺CD11c^{Hi} (Y-axis) analyzed for co-expression of MHC Class II (MHCII), CD14, CD11b, CD13, CD26 and CD205 (X-axis).

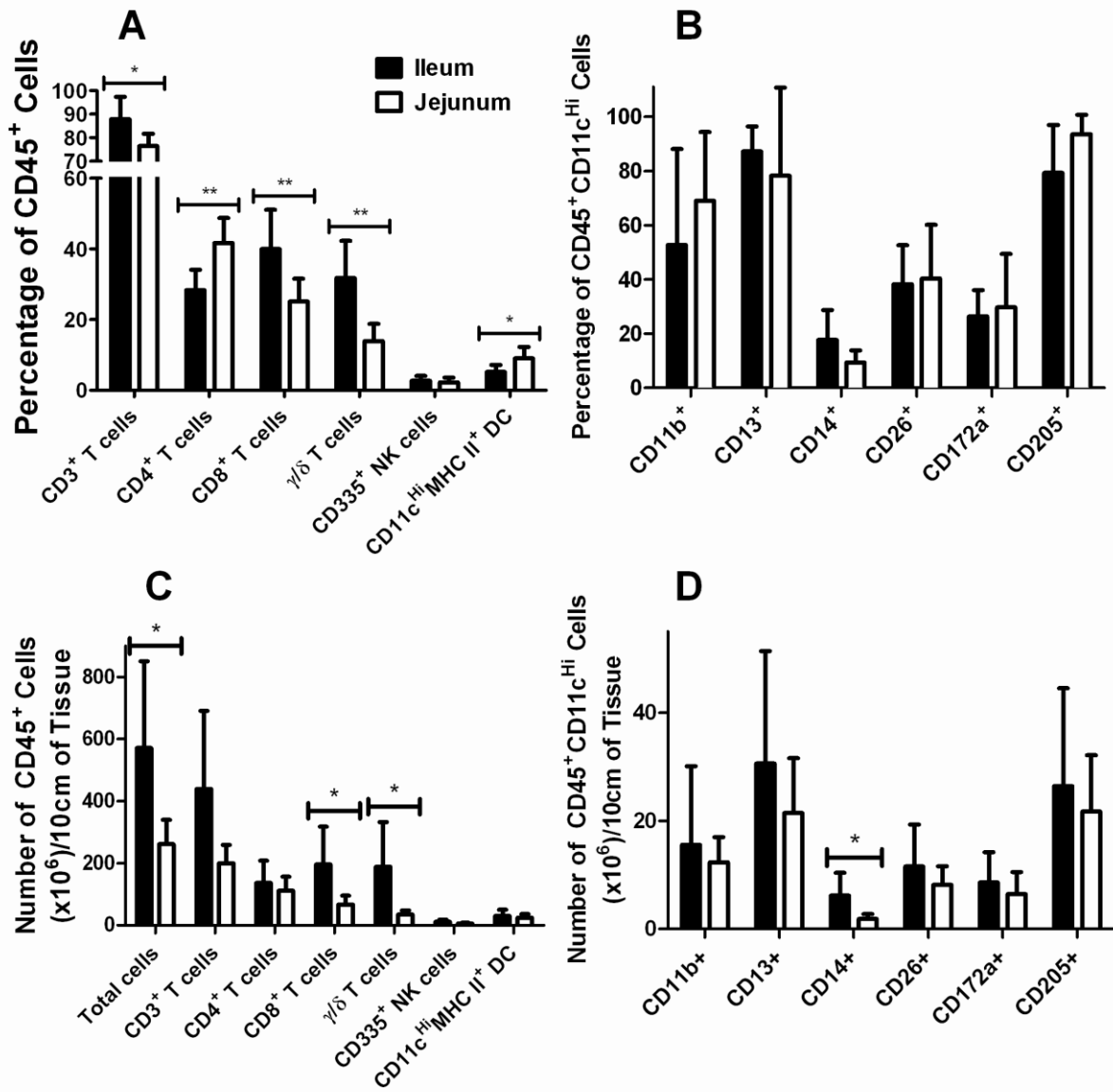


Figure 5.2. Frequency and number of mucosal leukocyte subpopulations isolated from ileum and jejunum of weaned calves. **(A)** Frequency of lymphoid and myeloid subpopulations expressed as a percentage of the total CD45⁺ population isolated from ileum and jejunum. **(B)** Frequency of myeloid subpopulation expressed as a percentage of the total CD45⁺CD11c^{Hi} population isolated from ileum and jejunum. **(C)** The number of lymphoid and myeloid cells isolated from a 10 cm segment of ileal and jejunal tissue was calculated based on the total number of CD45⁺ cells isolated. **(D)** The number of myeloid cells isolated from a 10 cm of ileal and jejunal tissue was calculated based on the total number of CD45⁺ CD11c^{Hi} cells isolated. Data presented are the mean and 1 SD of values for ileum and jejunum (n = 8). Significant differences between mucosal leukocyte populations in ileum and jejunum are indicated: * p < 0.05; ** p < 0.01.

The average yield of CD45⁺ leukocytes/10 cm segment of ileum (n = 8), after subtracting B cells, was 572 million cells (1 SD = 279 x 10⁶). In contrast, significantly (p < 0.05) fewer CD45⁺ leukocytes were recovered from a 10 cm segment of jejunum (n = 8) with an average yield of 262 million cells (1 SD = 79 x 10⁶). The absolute yield of lymphoid (Figure 5.2C) and myeloid cells (Figure 5.2D) from ileal and jejunal digests was calculated and the abundance of individual leukocyte populations was compared. This comparison confirmed CD8 and $\gamma\delta$ TcR T cells were numerically more abundant (p < 0.05) in mucosal leukocytes isolated from ileum but there was not a significant regional difference in CD4 T cell number. Furthermore, IHC confirmed that CD4 cells were abundant in the lamina propria of both the ileum and jejunum and absent from the mucosal epithelium (Figure 5.3). In contrast, both CD8 and $\gamma\delta$ TcR cells were abundant within the mucosal epithelium of both ileum and jejunum (Figure 5.3). The presence of CD4, CD8, and $\gamma\delta$ TcR cells in the isolated CD45⁺ mucosal leukocyte population confirmed that the tissue digestion has released both intraepithelial and lamina propria leukocytes.

There was no significant difference in the number of total myeloid cells (CD11c^{Hi}MHC II⁺) when comparing ileum and jejunum (Figure 5.2C). Calculating the absolute number of macrophage and DC subpopulations revealed that all myeloid subpopulations were similar in these two regions of the intestine with the exception of CD11c^{Hi}CD14⁺ cells which were significantly (p < 0.05) more abundant in the ileum (Figure 5.2D). IHC revealed a similar distribution pattern for CD11c⁺ cells throughout both the IEL and LP compartments of both ileum and jejunum (Figure 5.4; Table 5.2). It is interesting to note that CD13⁺ but not CD26⁺ cells were present within the IEL compartment of both ileum and jejunum. CD26⁺ cells were, however, found throughout the submucosa of the ileum but were rarely seen in the submucosa of the jejunum. In contrast, CD11b⁺ cells were restricted primarily to the LP of both ileum and

jejunum, although rare CD11b⁺ cells were detected within the ileal epithelium (Figure 5.4; Table 5.2). Staining for other macrophage markers, including both CD14⁺ and CD172⁺, revealed these populations were restricted to the LP and submucosa region of both the ileum and jejunum. Thus, regional differences in myeloid cell distribution were restricted to the occasional presence of CD11b⁺ cells in the ileal mucosal and the presence of CD26⁺ cells within the submucosa of the ileum.

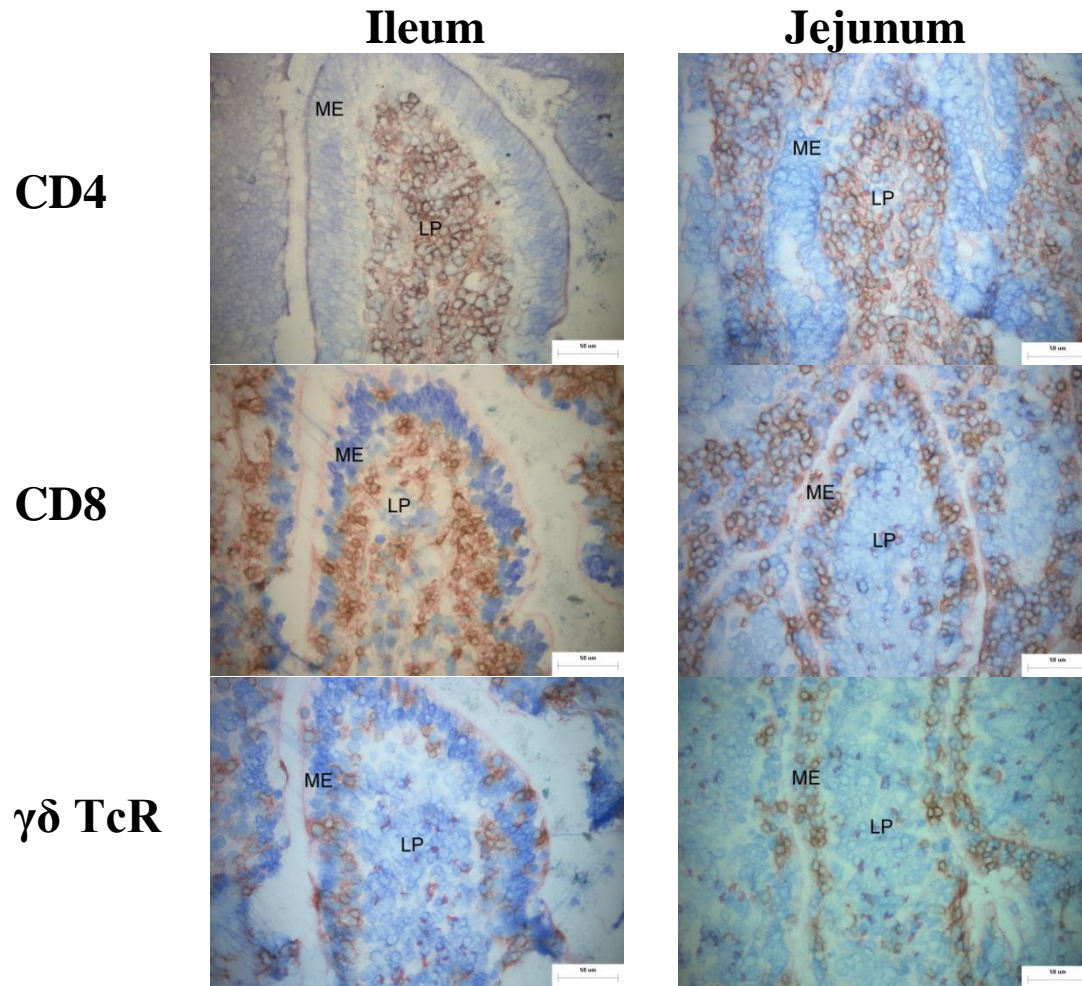


Figure 5.3. Immunohistochemical staining of cells expressing CD4, CD8, $\gamma\delta$ TcR in the ileum and jejunum of weaned calves. Tissue cryosections from three animals were stained with monoclonal antibodies specific for each leukocyte antigen and staining was visualized with 3,3'-diaminobenzidine substrate (brown cells). Irrelevant isotype-matched monoclonal antibodies were used to control for non-specific staining and no visible staining was observed. Representative images are magnified 40x and immune compartments are labeled in each sections: ME (mucosal epithelium); and LP (lamina propria).

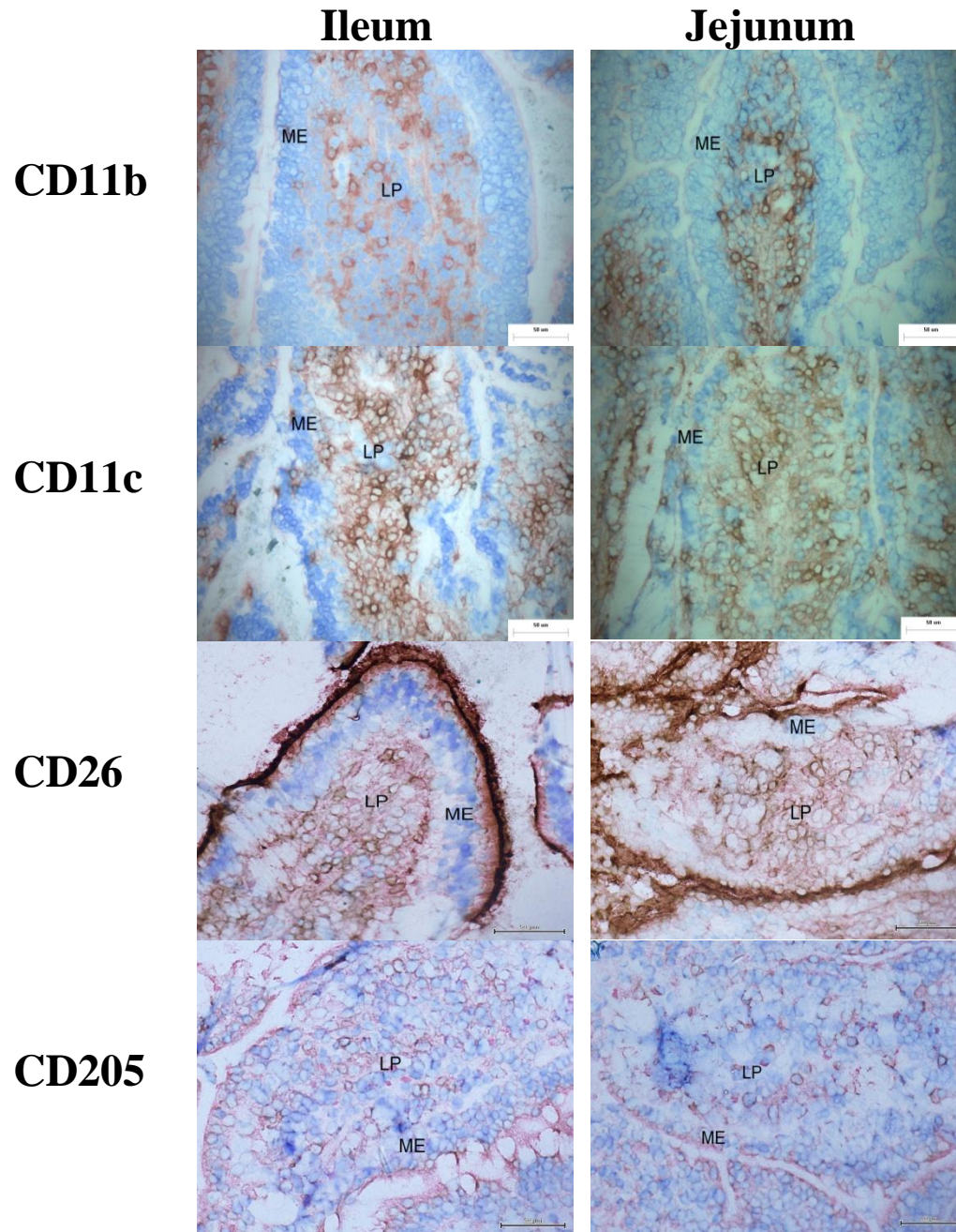


Figure 5.4. Immunohistochemical staining of cells expressing CD11b, CD11c, CD26, and CD205 in the ileum and jejunum of weaned calves. Tissue cryosections from three animals were stained with monoclonal antibodies specific for each leukocyte antigen and staining was visualized with 3,3'-diaminobenzidine substrate (brown cells). Irrelevant isotype-matched monoclonal antibodies were used to control for non-specific staining and no visible staining was observed. Representative images are magnified 40x. Immune compartments are labeled in each section: ME (mucosal epithelium); and LP (lamina propria).

Table 5.2: Distribution of lymphoid and myeloid cells in intestinal tissue of weaned calves.

Cell Type	ILEUM						JEJUNUM		
	IEL ¹	LP ²	DE ³	D ⁴	F ⁵	IF ⁶	IEL ¹	LP ²	SM ⁷
CD4 ⁺	-	+	R ⁸	+	R	+	-	+	-
CD8 ⁺	+	+	R	R	-	+	+	R	-
$\gamma\delta$ TcR ⁺	+	R	-	-	-	-	+	R	-
CD11b ⁺	R	+	+	+	+	+	-	+	R
CD11c ⁺	+	+	+	+	-	+	+	+	R
CD13 ⁺	+	+	+	+	+	+	+	+	-
CD14 ⁺	-	+	-	+	+	+	-	+	+
CD26 ⁺	-	+	+	+	+	+	-	+	R
CD172a	-	+	-	+	+	-	-	+	+

1. IEC: Intraepithelial leukocyte; 2. LP: Lamina propria; 3. DE: Dome epithelium; 4. D:

Dome region of PP follicle; 5. F: Lymphoid follicle in PP; 6. IF: Interfollicular region in PP;

7. SM: Submucosa; 8. R: Rare

5.4.2 Age-related Changes in Mucosal Myeloid and Lymphoid Populations

Development of the mucosal immune system is influenced by exposure to pathogens and commensal microflora [1] and previous investigations revealed a rapid increase in lamina propria (LP) T cell populations during the first week after calves were born but myeloid populations were not analyzed [118]. Fries et al reported, however, that the intestine of newborn calves was populated by distinct and diverse mucosal DC populations [20]. Therefore, we compared the relative frequency and abundance of mucosal lymphoid and myeloid populations isolated from the intestine of newborn and weaned calves. A comparison of ileal mucosal populations revealed a significant ($p < 0.001$) age-related increase in $CD3^+$ T cell frequency in weaned calves which was due primarily to a significantly ($p < 0.05$) greater CD8 T cell frequency (Figure 5.5A). Consistent with an age-related increase in the frequency of T cells was a significant ($p < 0.001$) decrease in the relative frequency of ileal $CD11c^{Hi}MHCII^+$ myeloid cells (Figure 5.5A). The yield of $CD45^+$ leukocytes/10 cm of ileal tissue increased significantly ($p < 0.05$) with age with an average yield of 343 million (1 SD = 183×10^6) and 572 million (1SD = 279×10^6) in newborn ($n = 9$) and weaned calves ($n = 8$), respectively. When mucosal leukocyte abundance was accounted for, the total ileal T cell number, including both CD8 and $\gamma\delta$ TcR T cells, increased significantly ($p < 0.05$) in older animals (Figure 5.5B). In contrast, adjusting for total leukocyte numbers revealed that $CD11c^{Hi}MHCII^+$ myeloid cell numbers remained unchanged in the ileum with increasing age.

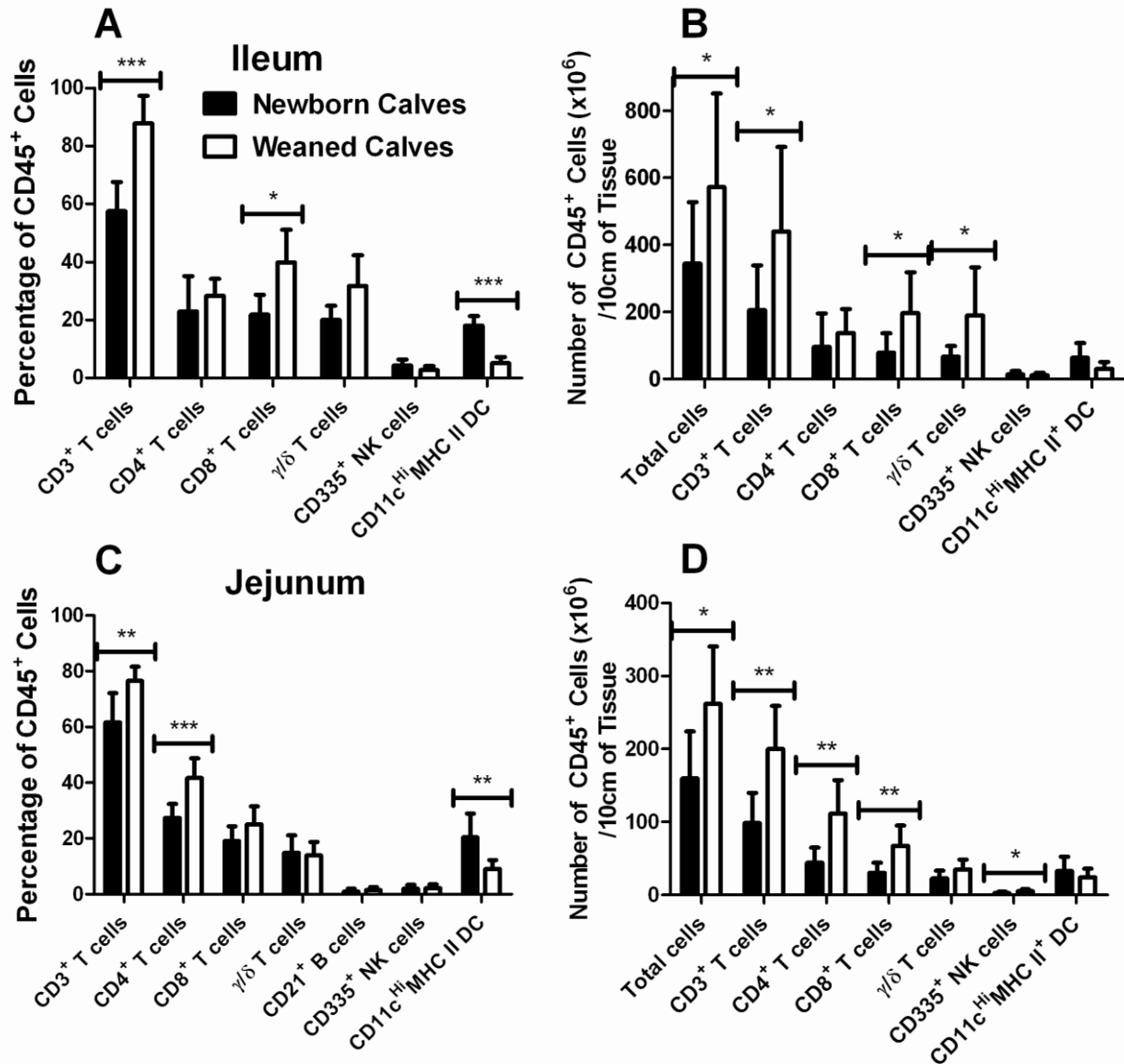


Figure 5.5. Comparison of the frequency and total number of mucosal leukocyte populations isolated from the ileum and jejunum of newborn and weaned calves. (A) Frequency of lymphoid and myeloid subpopulations expressed as a percentage of total CD45⁺ population isolated from the ileum of newborn and weaned calves. (B) The number of lymphoid and myeloid cells isolated from a 10 cm segment of ileal tissue collected from newborn and weaned calves was calculated based on the total number of CD45⁺ cells isolated. (C) Frequency of lymphoid and myeloid subpopulations expressed as a percentage of total CD45⁺ population isolated from the

jejunum of newborn and weaned calves. **(D)** The number of lymphoid and myeloid cells isolated from a 10 cm segment of jejunal tissue collected from newborn and weaned calves was calculated based on the total number of CD45⁺ cells. Data presented are the mean and 1 SD of values for ileal and jejunal tissue collected from newborn (n = 9) and weaned calves (n = 8). Significant age-related differences in ileal and jejunum mucosal leukocyte populations are indicated: * p < 0.05; ** p < 0.01; *** p < 0.001.

A significant ($p < 0.01$) age-related increase in CD3⁺ T cells frequency was also observed in the jejunum of weaned calves but only CD4 T cells appeared to contribute significantly ($p < 0.01$) to this change in frequency (Figure 5.5C). Also, as expected there was a relative decrease in the frequency of CD11c^{Hi}MHCII⁺ myeloid cells in the jejunum of weaned calves (Figure 5.5C). The yield of CD45⁺ leukocytes/10 cm of jejunum also increased significantly ($p < 0.05$) with age with an average yield of 160 million (1 SD = 64×10^6) and 262 million (1 SD = 79×10^6) cells for newborn and weaned calves, respectively. When cell number was calculated for various lymphoid and myeloid subpopulations recovered from jejunum then it became evident that both CD4 and CD8 T cells increased significantly ($p < 0.01$) with age (Figure 5.5D). Furthermore, when the change in mucosal leukocyte numbers was accounted for there was no significant age-related change in total myeloid (CD11c^{Hi}MHC II⁺) cell number (Figure 5.5D). It became apparent, however, that CD335⁺ NK cell abundance did increase significantly ($p < 0.05$) with age. Therefore, when both mucosal leukocyte frequency and abundance were analyzed it was apparent that the ileal and jejunal regions of the small intestine displayed unique age-related increases in T cell and NK cell numbers.

DCs play a key role in mucosal interactions with pathogens and commensal microflora and we previously observed significant differences in the distribution of DC and macrophage populations in the ileum and jejunum of newborn calves [20]. Therefore, we further investigated the distribution, frequency and abundance of individual mucosal myeloid subpopulations to determine if significant regional differences persisted with increased age. IHC had previously revealed unique distribution patterns for CD11c and CD11b myeloid cells in the ileal and jejunal mucosa of newborn calves. These differences in myeloid cell distribution were no longer apparent, however, in weaned calves (Table 5.2) but there was a significant ($p < 0.001$) increase

in the relative frequency of DC subpopulations (CD13⁺, CD26⁺, CD205⁺) in both the ileum (Figure 5.6A) and jejunum (Figure 5.6C). The ileal region was unique in having a significant ($p < 0.05$) decline in both the frequency (Figure 5.6A) and abundance (Figure 5.6B) of macrophages (CD14⁺, CD172a⁺). When DC abundance was calculated this revealed that DC number remained unchanged in the ileum with increasing age (Figure 5.6B). In contrast, the CD26⁺ DC subpopulation was unique in displaying significantly ($p < 0.05$) increased abundance in the jejunum with increasing age (Figure 5.6D). This myeloid subpopulation analysis confirmed that there were distinct regional differences when comparing the ileum and jejunum of weaned calves. Also, the regional differences apparent in weaned calves were distinct from those observed in newborn calves.

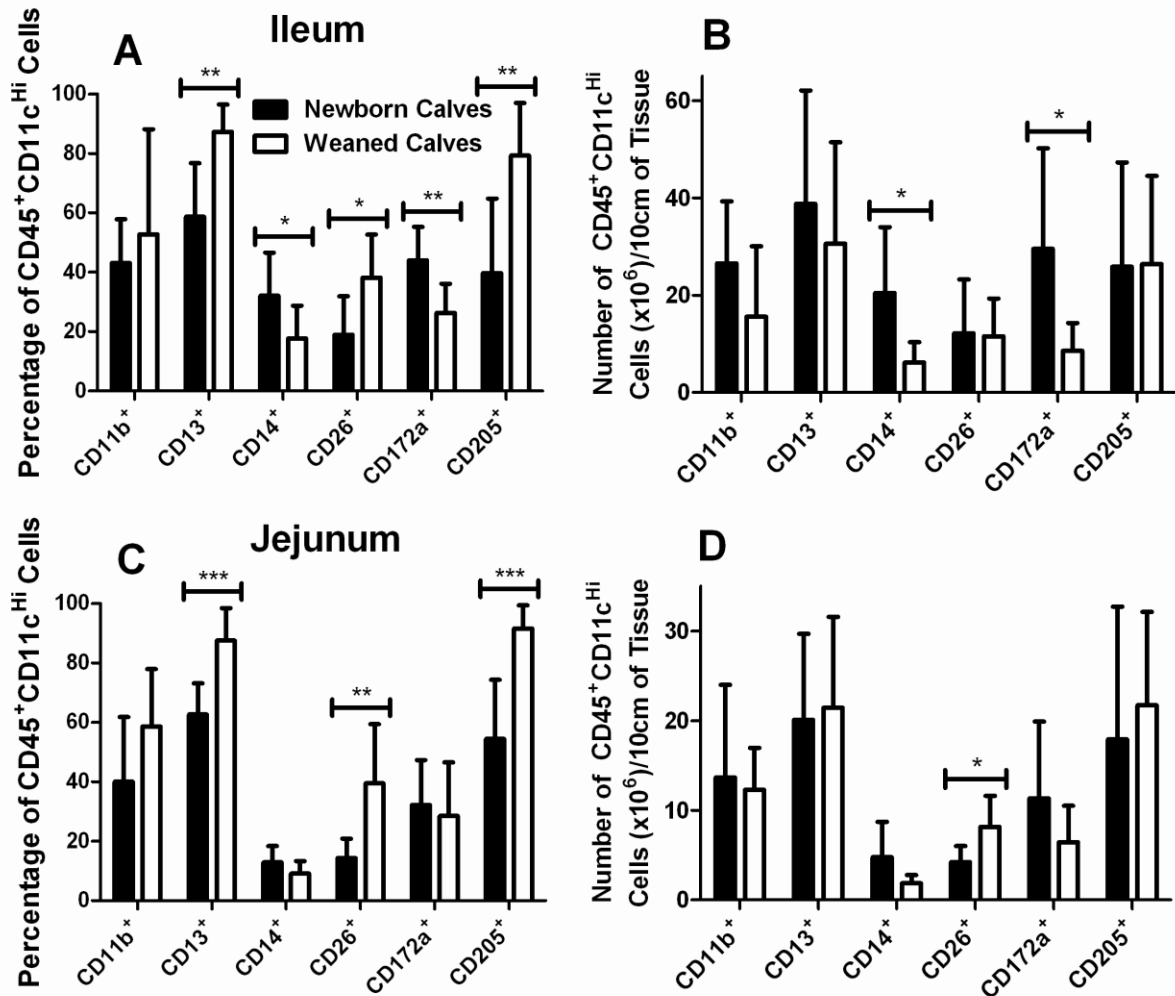


Figure 5.6. Comparison of the frequency and total number of mucosal myeloid subpopulations isolated from the ileum and jejunum of newborn and weaned calves. **(A)** Frequency of myeloid subpopulations expressed as a percentage of total CD45⁺CD11c^{Hi} population isolated from the ileum of newborn and weaned calves. **(B)** The number of myeloid cells isolated from a 10 cm segment of ileal tissue collected from newborn and weaned calves was calculated based on the total number of CD45⁺CD11c^{Hi} cells isolated. **(C)** Frequency of myeloid subpopulations expressed as a percentage of total CD45⁺CD11c^{Hi} population isolated from the jejunum of

newborn and weaned calves. **(D)** The number of myeloid cells isolated from 10 cm segments of jejunal tissue collected from newborn and weaned calves was calculated based on the total number of CD45⁺ CD11c^{Hi} cells isolated. Data presented are the mean and 1 SD of values for ileal and jejunal tissue collected from newborn (n = 9) and weaned calves (n = 8). Significant age-related differences in ileum and jejunum myeloid subpopulations are indicated: * p < 0.05; ** p < 0.01; *** p < 0.001

5.5 Discussion

Investigations in mice and humans revealed that phenotypically and functionally distinct mucosal DC subpopulations are located at specific sites throughout the GIT [138-142, 144-147, 154, 231]. These studies usually involved studying healthy individuals or age-matched healthy and diseased subjects. Age-related changes in mucosal leukocyte populations, especially myeloid subpopulations, have not been thoroughly investigated. Previously, we observed distinct mucosal DC subpopulations within the ileum and jejunum of newborn calves and also observed significant regional differences in both lymphoid and myeloid subpopulations throughout the small intestine [20]. One of the more remarkable regional differences was the restricted distribution of CD11c⁺ myeloid cells in the IEL compartment of the jejunum and CD11b⁺ myeloid cells in the IEL compartment of the ileum. Therefore, it was of substantial interest to determine whether the regional differences in mucosal leukocyte populations established in the newborn were maintained following sustained exposure to commensal microflora and possible exposure to pathogens. The present investigation confirmed that significant regional differences in mucosal leukocyte populations were present in the small intestine of older animals but these differences were distinct from those observed in newborn calves. These observations are consistent with the concept that environmental microbes and antigens have significant impact on the development of the mucosal immune system but these effects differ significantly throughout the GIT.

Regional differences between ileum and jejunum involved both lymphoid and myeloid subpopulations. It was critical, however, when analyzing these differences to not only compare cell frequency but to also calculate the abundance of individual cell populations since the overall number of mucosal leukocytes increased significantly with age. The tissue digestion method for

isolating mucosal leukocytes provides a minimal estimate of leukocytes numbers but previous analysis revealed cells were consistently released from both the IEL and LP immune compartments [20]. Therefore, tissue digestion of a consistent amount of intestinal tissue provided a representative sample of the major mucosal leukocyte populations. The actual increase in mucosal leukocyte populations in older animals is further compounded, however, by an increase in both intestinal length and diameter. Current values, therefore, substantially underestimate age-related changes in total mucosal leukocyte populations. Furthermore, mucosal leukocytes were isolated through the use of a 3 to 4 hour collagenase digestions. This prolonged enzymatic incubation may result in activation and altered expression of surface proteins by DCs and other leukocytes. IHC confirmed, however, that leukocyte subpopulations identified within flow cytometry were actually located within the mucosa and lamina propria of the small intestine and the same tissue digestion protocol was consistently used throughout all studies.

Despite the limitations of our sampling methodology, it was evidence that regional differences were present within the ileum and jejunum when analyzing age related changes in mucosal leukocyte populations. Changes in T cell subpopulations were of particular interest since the ileal region was unique in having significantly greater CD8 and $\gamma\delta$ TcR T cell numbers in older calves (Figure 5.2C). Many bovine $\gamma\delta$ TcR T cells co-express CD8 so it is not surprising to see a coincidental increase in these two T cell subpopulations [213]. It was somewhat surprising, therefore, that a significant age-related increase in the number of CD4 and CD8 T cells in the jejunum was not associated with a significant increase in $\gamma\delta$ TcR T cells (Figure 5.4). The large increase in CD4 T cells in the jejunal region (Figure 5.5) also raises interesting questions regarding the function of these cells since the small intestine is an important site for the generation of Foxp3⁺CD4⁺CD25⁺ T regulatory cells [162]. Future studies analyzing the

phenotype and function of mucosal CD4 T cells will be important to determine if there are regional differences in the generation of T regulatory cells and if these differences have a direct impact on mucosal inflammation and systemic immune responses. Finally, the difference between jejunal and ileal lymphoid populations was further highlighted by a significant age-related increase in NK cell numbers in the jejunum but not the ileum (Figure 5B & 5D). This observation suggests that significant regional differences in innate mucosal immune defences may influence interactions with enteric pathogens. This is an intriguing possibility when considering pathogens which infect specific sites within the GIT. For example, *Mycobacterium avium* subspecies *paratuberculosis*, preferentially infects the terminal small intestine in cattle.

Through the use of flow cytometry we identified a relatively large population of CD11c^{Hi}MHCII⁺ myeloid cells in the ileum and jejunum (Figure 5.2C). Co-staining for CD11c and a variety of macrophage and DC markers confirmed the presence of diverse populations of myeloid subpopulations in the ileum and jejunum which were well delineated with flow cytometry (Figures 5.1C and 5.2C). Despite dynamic age-related changes in mucosal T cell populations the total number of myeloid cells (CD11c^{Hi}MHC Class II⁺) remained relatively stable as calves increased in age (Figure 5.5B & 5.5D). The abundance of macrophages and DCs in the small intestine of newborn calves suggests that neonatal mucosal immune system has substantial capacity to sample and present enteric antigens. It was only when individual myeloid cell subpopulations were analyzed that significant regional and age-related changes became apparent for both the macrophage and DC subpopulations. The only significant difference between the ileum and jejunum in weaned calves was a greater abundance of CD14⁺ macrophages in the ileal region (Figure 5.2B & 5.2D). This regional difference may be due to the presence of CD14⁺ cells within the dome, follicular, and inter-follicular regions of the ileal PP

(Table 5.2) which is absent in jejunal tissue. Thus, myeloid populations became more similar throughout the small intestine with increasing age. This was also apparent when using IHC to analyze the distribution of individual myeloid subpopulations within various mucosal immune compartments. We previously observed that CD11c and CD11b myeloid cells had different patterns of distribution in the ileal and jejunal mucosa of newborn calves [20]. In contrast, CD11c⁺ cells were present and CD11b⁺ cells were largely absent throughout the mucosa of both the ileum and jejunum in weaned calves. Further studies will be required to determine whether these changes in myeloid cell distribution alter the mucosal immune system's capacity to sample luminal contents including commensal microflora and pathogens.

Another consistent age-related change observed throughout the small intestine was a significant increase in the frequency of CD13⁺, CD26⁺, and CD205⁺ DC populations (Figure 5.6A & 5.6C). There was, however, a unique regional change in myeloid populations with the ileum which displayed an age-related decline in the frequency and number of macrophages (CD14⁺ and CD172a⁺; Figure 5.6A & 5.6B). This observation must be interpreted within the context that macrophages are abundant within the lymphoid follicles of the ileal PP and an age-related decline in PP development may influence the cell populations isolated in our tissue digests [232]. The jejunum also displayed one unique age-related change in DC subpopulations with a significant increase in CD26⁺ DCs in older animals (Figure 5.6D). A consistent tissue digestion protocol was used throughout all studies so the observed changes in DC subpopulations should not be an artifact of our cell isolation procedure. Further studies with isolated mucosal CD26⁺ DCs will be required, however, to determine if this DC subpopulation possesses unique functional properties important for host interactions with pathogens or commensal microflora. These studies will determine if the neonatal mucosal immune system has a significantly different

capacity to recognize pathogens, initiate innate immune responses, and induce acquired immune responses. This knowledge is critical for understanding enteric infections in the newborn and designing potential mucosal vaccination strategies.

5.6 Conclusion

The mucosal immune system continues to be an area of critical interest for understanding disease pathogenesis, immune regulation, and mucosal vaccine development. This is the first study to analyze age-related changes in mucosal lymphoid and myeloid cell populations of the small intestine. As the mucosal immune system encountered an increasing number of pathogens within the context of normal commensal microflora, there was a significant increase in mucosal leukocytes, characterized by increased T cells and an enrichment of mucosal DCs. Furthermore, these age-related changes varied throughout the small intestine which suggests significant regional differences exist for both innate and acquired immunity. Thus, age-dependent development of the mucosal immune system may be an important factor determining differences in host responses to infection and commensal microflora at specific sites throughout the gastrointestinal tract.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

The mucosal surface of the small intestine is an important interface between the environment and the host immune system. Very little is known, however, regarding mucosal macrophages and DCs in the newborn and how these populations change as animals are exposed to dietary antigens, commensal microflora, and pathogens. The overall goal of my project was to address the hypothesis that distinct myeloid cell populations are present in the small intestine of newborn calves and that these populations differ significantly in different regions of the small intestine and change with age. To address these questions I first developed the methodology to isolate and characterize mucosal myeloid subpopulations. The isolated myeloid subpopulations were then compared between newborn (3-5 weeks old) and weaned calves (6 months old).

Techniques employed to achieve these goals include flow cytometry, cytopins, IHC, and cell sorting using flow cytometry. Since LPL originate in the bone marrow and traffic through the blood, we also isolated PBMC. This comparison allows us to investigate whether specific DC subpopulations are present in the blood and then traffic to the LP or whether DC subpopulations differentiate following recruitment to the intestine.

Three colour flow cytometry was used to phenotypically characterize LPL and PBMC in blood. In the ileum and jejunum our digestions recovered epithelial cells as well as LPL. I, therefore, recognized the need to use a leukocyte-specific marker (CD45) to exclude epithelial cell contamination from my analysis following tissue digestion. Due to the presence of B cell follicles in the ileal PP, we also had to mathematically subtract the B cells from the analysis of the ileal mucosal leukocyte populations. The jejunal sections that were sampled were void of PP

and thus were not affected by B cell contamination. All cells isolated from the blood are leukocytes and thus did not require the use of a leukocyte-specific marker.

IHC was used in the ileum and jejunum to reveal the tissue distribution of different cell types. Important information was gained from these data such as the locations of CD4, CD8, CD11b and CD11c cells. Comparing newborn and older animals also shows us how the locations of cell populations change with age, which may affect their role in the tissues. Using flow cytometry and IHC concurrently gives us a much more complete picture of the development of the LPL in the ileum and jejunum.

Similar research has been conducted in other species, including mice and sheep. Few studies have, however, investigated development of mucosal myeloid cell populations in the neonate. The knowledge obtained through the present study is important since it establishes baseline values for normal healthy calves at two different time points. More specifically, knowledge gained about the neonatal mucosal immune system can be used to investigate responses to neonatal vaccines, neonatal infections, and therapeutic approaches involving the neonatal enteric mucosal immune system. These studies provide new insight into the mucosal immune system of calves.

Mucosal lymphocyte and myeloid populations from the ileum and jejunum of newborn calves were characterized shortly after birth to determine if distinct mucosal DC subpopulations were present at this time. Although there were similar DC subpopulations in both the ileum and jejunum of newborn calves, regional differences were discovered in the frequency and distribution of leukocytes in the IEL and LPL compartments. There were differences in the CD11c^{Hi}CD14⁺ as well as the CD335⁺ NK cell population. T cell and DC subpopulations are present in newborn calves, which should support the induction of acquired immune responses to

various antigens. IHC revealed that CD11c⁺ cells were found in the mucosal epithelial layer in the jejunum but not in the ileum. This difference in cell distribution raises interesting questions whether immune surveillance may differ between the ileum and jejunum. Future studies will be required to further define the phenotype and function of the CD11c⁺ located within the jejunal epithelium.

When the mucosal immune system was analyzed in weaned calves a number of unique aspects were observed. The number of CD8, $\gamma\delta$ TcR T cells and CD11c^{Hi}CD14⁺ cells were greater in the ileum than the jejunum. Furthermore, CD11c⁺ and CD11b⁺ myeloid cell distribution was now similar throughout the mucosal epithelium of the ileum and jejunum. In weaned animals, both the number and frequency of all T cell subsets increased considerably when compared to newborn calves. CD11c^{Hi} MHC Class II⁺ myeloid cells decreased in frequency with age, however, the number of total myeloid cells remained constant. In particular, CD13⁺, CD26⁺, and CD205⁺ DCs were enriched with age, while CD14⁺, CD172a⁺ DCs decreased. The increased number and frequency of T cells suggests there are considerable age-dependent changes in the acquired immune system. In contrast, the relatively constant number of DCs with increasing age suggests that the innate immune system may be fully competent in the newborn animals. Functional studies of isolated mucosal DC subpopulations will be required to determine if there are age-related changes in DC function.

There were also distinct differences in DC phenotype and frequency when comparing jejunal and blood cell populations. Most notable was the absence of CD11c^{Hi}CD13⁺ cells in blood and significant differences were observed in many other myeloid populations. In comparison to blood, there was significant DC enrichment in the intestine of newborn calves. This observation supports the conclusion that local DC differentiation plays a role in determining

the phenotype of mucosal DC populations present in the small intestine. Furthermore, the recruitment and differentiation of DCs does not appear to be influenced by increasing age and exposure to either commensal microflora or pathogens. Further studies in fetal calves or calves within the first few days of life may be required to more clearly determine if DC recruitment and development is truly independent of environmental influences.

Weaned calves have undergone significant dietary and physiological changes with prolonged exposure to commensal microflora. These changes include morphing from a monogastric to a ruminant with dietary changes from milk to plant-based material. This dietary change is also associated with significant changes in the commensal microflora population, which can potentially affect the mucosal immune system. Comparing these two age groups of calves gave us a glimpse into how the mucosal immune system reacts to these different environmental stimuli.

The number of LPL recovered from weaned animals was significantly greater than those recovered from newborn calves. In the ileum this increase was reflected primarily in CD8⁺ T cells. In the jejunum this increase was reflected in CD4⁺, CD8⁺ and $\gamma\delta$ TcR T cell populations. The frequency of CD11c^{Hi} MHC Class II⁺ cells decreased over time, but the number of CD11c^{Hi} MHC Class II⁺ cells remained constant. It is possible that at 3 weeks of age calves the mucosal immune system has acquired sufficient myeloid cells to provide adequate antigen presenting capacity to respond to environmental challenges. A full assessment of DC function in the neonate and older animals will require the isolation and purification of individual DC subpopulations. Further functional studies can then be performed to determine if the apparent stability in mucosal DC subpopulations with increasing age reflects a full capacity to sense pathogens and present antigen.

In conclusion, these studies confirm the presence of regional differences in the intestinal mucosal immune system. Furthermore, many of these regional differences are age-dependent which suggests that the development of the mucosal immune system may be shaped by a variety of environmental factors. The phenotypic differences in regional mucosal leukocyte populations also suggest there may be regional differences in the innate and acquired immune systems. Further studies in this area could provide insight into how and why pathogens interact with the host within specific regions of the small intestine. A better understanding of the age-related changes in the cellular composition and functional capacity of the enteric mucosal immune system could also have important implications for improving oral vaccine delivery.

CHAPTER 7

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